

Risk Indicators of Dental Erosion in Experimental Mouse Models and Humans

A Doctoral Thesis by

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List of Papers

The following papers (I-III) are submitted in partial fulfillment of the requirements for the degree Philosophiae Doctor (Ph.D.) at the Faculty of Dentistry, University of Oslo, Oslo, Norway. The present thesis is based on experimental work carried out at the following departments: Department of Oral Biology, Faculty of Dentistry, University of Oslo, Oslo, Norway; Nordic Institute of Dental Materials (NIOM AS), Oslo, Norway; Department of Oral Biology, School of Dental Medicine, University of Pittsburgh, Pittsburgh, PA, USA. The papers are referred to by their Roman numerals throughout the text.

- Paper I **Tulek A**, Saeed M, Mulic A, Stenhagen KR, Utheim TP, Galtung HK, Khuu C, Nirvani M, Kristiansen MS, Sehic A. New animal model of extrinsic dental erosion-Erosive effect on the mouse molar teeth. *Arch Oral Biol* 2018; 96:137-145.
- Paper II **Tulek A**, Mulic A, Stenhagen KR, Galtung HK, Saeed M, Utheim TP, Khuu C, Galteland P, Sehic A. Dental erosion in mice with impaired salivary gland function. *Acta Odontol Scand* 2020; doi:10.1080/00016357.
- Paper III **Tulek A**, Vieira AR, Weber ML, Bezamat M, Deeley K, Stenhagen KR, Sehic A, Søvik JB, Mulic A. Aquaporins` influence on different dental erosive wear phenotypes in humans. *Caries Res* 2020; doi:10.1159/000505965.

Abbreviations

AQP	Aquaporin
BEWE	Basic Erosive Wear Examination
DNA	Deoxyribonucleic acid
GER(D)	Gastroesophageal reflux (disease)
GWAS	Genome wide association study
NOD	Non-obese diabetic
PCR	Polymerase chain reaction
PDS	Public dental service
SEM	Scanning electron microscope
SNV	Single nucleotide variant
VEDE	Visual Erosion Dental Examination

Introduction

The World Health Organization (WHO) defines oral health as “a state of being free from chronic mouth and facial pain, oral and throat cancer, oral infection and sores, periodontal disease, tooth decay, tooth loss, and other diseases and disorders that limit an individual’s capacity in biting, chewing, smiling, speaking, and psychosocial wellbeing” [1]. Oral health is an integral part of overall health; hence, it affects many aspects of one’s general well-being. In the past, dental caries and periodontal disease have been subjects of concern for both researchers and clinicians, whereas dental erosive wear was not considered as an important condition, mainly due to a lack of knowledge. However, today it has emerged as one of the current matters of many discussions of oral conditions, mostly because of the increasing prevalence and incidence [2, 3].

Physiological wear of the dentition is present throughout the lifetime, influenced by various mechanical and chemical challenges. As long as their effect is mild and they are in balance with the host response, the loss of dental substance is often considered as physiological (functional). A disorder (condition) develops when the impact of the influencing factors becomes exaggerated and the host response is inadequate. Nevertheless, the etiology of dental erosive wear is complex and far beyond just the mechanical tooth wear facilitated by acids. Today, there is a significant number of both theoretical and clinical evidences to assign the multifactorial origin of this condition, even though some aspects of the complex etiology have still not been completely unveiled. Several studies have emphasized a broad range of variations in dental erosive wear among individuals with the similar level of acidic exposure, suggesting a varying susceptibility of the host [4, 5]. Based on these findings, additional studies devoted to the investigations of the influence of genetic contribution emerged, in order to find the missing puzzles [6, 7]. Therefore, it is mandatory to explore further the heritability in order to

gain more knowledge. The fact that a number of dental erosive wear patients remains undiscovered for a long time represents an additional challenge, as it is not an easy task to detect the initial symptoms, for the patient itself, as well as for the dental practitioner. When not diagnosed at the initial phase and treated effectively, erosive wear can lead to exposure of dentine.

The first symptoms of dental erosive wear reported by patients are often increased sensitivity of the teeth and pain. The loss of physiological contacts caused by morphological changes of the teeth may result in imbalanced occlusion, and, in extreme cases, it can affect the physiology of the temporomandibular joint. Taking into consideration these facts, one can perceive the importance of prompt and proper actions. Among many aspects of management, the preventive approach is of great importance. However, platforms for development of preventive strategies are built on the fundamental knowledge about the host susceptibility, risk factors, and the mechanisms of their interplay. The importance of empowering the research community and dental profession with this information about this condition has become visibly clear. For this broad research area, creating a model of disease may represent a basis for further research, especially since *in vivo* studies including humans with subsequent loss of dental substance strongly violate the principles of human research ethics. Therefore, the need for a standardized animal model for studying dental erosions, where the salivary influence and soft tissue interactions resemble to that in a human oral environment, is warranted.

Dental Erosive Wear

The term *erosion* is derived from the Latin verb *erodere, erosi, erosum* (to gnaw, to corrode), and it is used to describe the process of gradual loss of the object's surface. The terms *dental erosion* (DE), *dental erosive wear* (DEW) and *erosive tooth wear* (ETW) have been repeatedly used in the literature to refer to the same phenomenon. The term dental erosion is describing solely a chemical process of dental hard tissue loss, without involvement of bacteria, when the surrounding aqueous phase is undersaturated with respect to tooth mineral [8]. The terms dental erosive wear and erosive tooth wear refer to the chemical dissolution and softening of the tooth substance joined with mechanical wear, such as attrition and abrasion [9-11]. These terms are broadly used to refer to erosive loss of dental tissues since these factors act simultaneously in the oral cavity [12].

The earlier data about dental erosive wear in humans stem from the examination of teeth from archeologically obtained skulls, and there are findings supporting the existence of dental erosion even in primitive populations [13]. However, from an anthropological point of view, tooth wear was considered pathological only when the function of the tooth was impaired [14]. Therefore, it is likely to believe that dental erosion may have been overlooked in the early studies of tooth wear. G.V. Black, the founder of modern dentistry, described already in the beginning of 1990s dental erosive wear as a disease with a plausible multifactorial etiology and increasing prevalence [15]. The interest in dental erosion and its role in the wear of the dentition did not increase considerably until the mid-1990s. The turnover occurred mostly due to the changing focus from studies investigating tooth wear in the adult population, to investigations in children and adolescents, as well as from emphasizing the importance of etiological factors resulting in dental erosive wear [16]. The modern lifestyle carries some essential changes regarding the individual's habits. Namely, some food and drinks have a high content of acids.

The accessibility and the frequency of the consumption of such acidic products particularly among children and adolescents has increased [17]. This contributes to an increased risk of development of dental erosive wear. It is therefore reasonable to improve the knowledge about dental erosive wear, and to further investigate the complex interplay between the main risk factors and the additional factors for this condition.

Prevalence

To estimate the prevalence of dental erosive wear is not a simple task. Some of the challenges are large variations in indices, different population categories and sample sizes, as well as the techniques of recording. In addition, the choice of reference teeth, calibration of the examiners and quantification of dimension of tissue loss are still heterogeneous. This may be an explanation for the large difference in prevalence reported worldwide. Even so, it is still possible to draw some valuable conclusions about the severity of this condition. However, most prevalence data are available from European studies.

In 2013, a study comprising seven European countries was conducted with the aim to investigate the prevalence of dental erosive wear over the continent. Buccal and oral surfaces of the teeth from individuals of 18-35 years of age were clinically examined. Erosive wear was detected in 57.1% of the study population (N=3187), and the highest level was found in patients from the United Kingdom (54.4%). Factors such as reflux, frequent vomiting, fresh fruit and juice consumption were associated with the presence of erosions. As the presence was identified in the large part of the population, it was concluded that tooth wear is a common problem [18].

Several studies have investigated the prevalence of erosive wear in Norwegian populations [19-21]. Mulic at al. assessed the prevalence among 18-year-old adolescents in

Oslo, Norway. Out of the total sample (N=1456), 38% of the participants exhibited signs of erosive wear. Male subjects were identified with significantly more erosions, and more severe erosive lesions than female subjects [19]. Two years prior to this study, a group of 319 adolescents of 16 years of age, from Tromsø, Norway was examined using clinical intraoral photographs and Visual Erosion Dental Examination score (VEDE) in order to determine the prevalence and severity of dental erosions. Dental erosive wear was present in almost 40% of the population and 93% of the patients with detected erosions had “cuppings” on the molars [20]. Søvik and colleagues examined a population of 795 adolescents, 16-18 years of age, from the western part of Norway and found presence of dental erosive wear in 59% of the individuals. Cuppings on the molars were present in 66% of the population who exhibited erosive wear [21].

An investigation of the prevalence of dental erosions in Sweden has reported 11.9% among 13-14 year-olds, 22.3 % among 18-19 year-olds [22] and 75% among 20 year-olds, out of which 18% were extensive erosions (three or more molars with “cuppings” and/or presence of erosions on maxillary incisors) [23]. A Finnish study from 2016 investigating erosive wear in 1962 participants born in 1966 showed that nearly one of ten individuals suffered from severe erosions exhibiting a need for a restorative treatment [24].

Incidence

So far, a few studies have investigated the incidence or progression of erosive wear. However, the results obtained have in general been homogenous. Lussi and Schaffner investigated a group of 55 adults divided in two age groups (26-30 and 46-50 years), over 6 years, and reported progression of erosive wear on facial and occlusal surfaces [25]. They observed that the incidence was more noticeable in the older age group. Ganss et al. observed an incidence of 17.3% among children, mean age 11.4 ± 3.3 , using orthodontic models [26]. In 2003, the

incidence of erosive wear of 12.3% was reported over a 2-year period in a group of 12-year-old children of mixed ethnicity [3]. El Aidi et al. studied both incidence and progression of erosive wear in 622 children aged between 10 and 12 years during the period of 3 years. The incidence decreased from 26.5% at 11 years to 6.4% at 14 years [27]. In 2017, the prevalence and severity of dental erosive wear of Norwegian 16-18 year-olds registered with erosions in 2012 and 15 year-olds registered with erosions in 1985 was compared. At that time-point, the condition was found to be more severe when comparing to the data obtained thirty years earlier [28].

Etiology

Many factors play a role in the onset and progression of dental erosive wear. It has generally been accepted that this is a condition with a multifactorial etiology [2, 16]. Therefore, it is challenging to classify these potential risk factors with regard to development and progression. The present thesis attempted to clarify some of those potential factors by studying both risk factors and risk indicators related to dental erosive wear. The term *risk factor* refers to an environmental, biological or behavioral factor that contributes to the probability for a disease to occur, while its absence reduces this probability. Risk factors are usually confirmed in longitudinal studies [29]. However, a *risk indicator* is a potential risk factor, commonly established in cross-sectional studies that needs to be confirmed by longitudinal studies [29]. Dental erosion develops in the presence of an acid source, either extrinsic and/or intrinsic [30]. Common extrinsic acid sources are acidic food, drinks and medications, whereas the intrinsic source is regurgitation of gastric acid. The protons of the acidic agent affect the components of hydroxyapatite such as carbonate, phosphate and hydroxyl ions, resulting in dissolution of the hydroxyapatite crystals. In addition, factors like the oral environment and genetic susceptibility

are of utmost importance. However, it is likely to believe that these factors interact interchangeably in the function of time.

Nutritional Factors

Acidic Food

It has been observed that the prevalence of dental erosive wear is increasing, predominantly in the younger age groups [28, 31]. A shift in nutritional habits and lifestyle is the main cause for this [32, 33], as well as the factors that influenced these nutritional habits and lifestyle to change. Exposure of children to sour taste at an early age increases the preference for acidic food and drinks later in life [34]. Acidic fruits and beverages are today easily available throughout the whole year. New types of sour candies are accessible in the market, and children are exposed to intense advertising of these products.

Research in the mid last century have indicated the harmful effect of acids to the dental tissue, and the erosive activity of acids as ingredients of food and drinks has been demonstrated in several studies [35, 36]. Case control, cross-sectional studies and numerous case reports have also showed diet to be an important etiological factor for the development and progression of erosions. In 1985, a significant number of erosions in individuals on lacto-vegetarian diet was found [37]. Investigation of nutritional factors has shown that, among others, considerable risk of erosion existed when citrus fruits were eaten more than twice a day [38]. El Aidi et al. found sour vegetables and vitamins to be significantly associated with the incidence of erosive tooth wear [39]. A study in 2004 found pickles, vinegar, salt and vinegar crisps and brown sauces to be erosive [40]. In addition, sour sweets have been identified as a risk factor [41, 42]. It has been anticipated that the main cause for erosive attack stems from the fact that sour sweets have very acidic pH and prolonged oral contact. One of the dietary products which is commonly misunderstood is yoghurt. Namely, due to its low pH of 4.0, it is considered erosive. However,

owing to its high mineral content (calcium and phosphate) it is not found to be harmful towards the dentition [39, 43].

Acidic Drinks

Similarly to the growth in the acidic food intake, the consumption of acidic drinks has also increased significantly over the past few decades [43]. Assessment of the dental erosive wear in some individuals has shown a strong association with high consumption of acidic drinks. Dugmore and Rock suggested the high consumption of carbonated drinks as a prognostic factor for the amount of dental erosion [44]. A study among Swedish children showed positive correlation with the severity of dental erosion in the 18-19 and 13-14 age groups. However, in the 5-6 year old group there was no association found [45]. A study of 14-year-old children in England revealed that over 80% regularly consumed soft drinks. More than 10% had over three intakes per day. When these data were combined with the prevalence of dental erosion, a statistically significant correlation was found [46]. In 157 children, age of 12-14 years, in Saudi Arabia, the overall prevalence of dental erosions was 66.9%, showing a strong association with carbonated drinks and herbal hibiscus drink [47]. Okunseri et al. found a positive association between children affected with erosive wear, and their consumption frequency of apple juice [48].

Energy drinks are alleged to increase the energy level and enhance physical and mental performance. They are used as dietary supplements, with a high prevalence especially among younger individuals [49]. According to the Global Energy Drinks Market report it is estimated an increase of 7.1% in production of energy drinks worldwide in the period from 2019 to 2024 [50]. Due to their chemical properties, they are considered as erosive and are a significant factor for inducing dentine hypersensitivity [51, 52]. Sports drink is another vastly popular beverage among young individuals, particularly among those individuals performing sports activities, as

a fluid and electrolyte replenishment. They contain acid and have a low pH. Therefore, they are considered as potentially erosive towards dental tissues [53]. It is assumed that the intake of a sports drink in combination with xerostomia caused by dehydration increases the chance of erosions development. Although sports drinks are mostly consumed during the training, it was found a high consumption among the US high school students who spent two or more hours watching television [54]. A British study from 2016 concluded that sports drinks are consumed regularly among adolescents and outside the physical activities [55].

It has been noticed that not only the amount and frequency of acidic drinks have effect on development of dental erosions. Different drinking habits, such as swishing or holding drinks in the mouth is also of importance [56]. Johansson et al. found a high level of erosion correlated with retaining manner of drinking, i.e. keeping or swishing the drink in the mouth for a certain time prior to swallowing [57]. However, another study found no association between the manner of drinking (swilling the drink in the mouth vs. swallowing straight away) and the presence of dental erosive wear [42]. The same study found that the consumption of beverages with the use of a straw was associated with less dental erosive wear prevalence when compared to drinking directly from a bottle.

Consumption of Acidic Drinks in Norway

For the purpose of this thesis, a separate section is used to give an overview over the current data of acidic drinks consumption in Norway. Norway is one of the top European countries when it comes to intake of acidic drinks, such as juice and soda [58]. A research study among 1500 Norwegian adolescents and their parents, recorded the intake of sugar-sweetened beverages, and found this to be low during working days, but doubled during the weekend [59]. Detailed investigation of the drinking pattern of acidic beverages in Norway has found that, on average, 34% of the participants were consumers of sugar-sweetened beverages, with the

proportion being higher for men, and the average consumption amount was about 4 dl per day. Wine intake was higher on weekend, with 16% and 17% of average in men and in women, respectively. Participants being overweight had higher results in consuming artificially sweetened beverages, compared to participants with normal or low body mass index. Additionally, people interested in a healthy diet had 21% lower odds of consuming artificially sweetened beverages, compared to people with moderate, low or no interest in healthy diet. The oldest age group had 76% lower odds of sugar-sweetened beverage consumption compared to the youngest participants [60]. Official data from 2018 showed that it was sold over 46 million liters of soda drink in the country during that year. The same trend continued in 2019 [61]. Regarding energy drinks a total amount of 40 million liters was consumed in 2019 [62]. Energy drinks are not recommended for adolescents of 15 years of age or younger, in Norway. Still, the number of children and adolescents who reported drinking energy drinks on a daily base has increased in the last few years. The majority of the teenagers are in the age group from 16-18 years, however, it is important to notice that around 20% of children aged from 10-12 years frequently consume energy drinks as well [63]. One study found that 52.3% of the Norwegian secondary school students (N= 31,091) aged from 12-19 years are frequent consumers of energy drinks [64].

Host Factors

Saliva

Saliva is a significant biological factor in maintaining individual's oral health. An association between the amount of secreted saliva in the oral cavity and the incidence of oral caries has been found [65]. Diminished salivary flow is caused by hypofunction of salivary glands and it can affect the individual's oral health. The symptoms of dry mouth comprise thirstiness, sensations of dry tongue and throat, sticky feeling in the mouth, bad breath (halitosis), burning

sensations in the mouth, difficulty speaking, chewing and swallowing, as well as increased plaque [66]. The very substantial role of saliva in the development of dental erosions has been observed. Natural saliva is an essential factor in the prevention of demineralization and facilitation of remineralization of dental tissue, as saliva contains inorganic components. Physiological flow rate is of high significance in the management of dental erosive wear, as it has been shown that diminished salivary flow is the cause of inadequate protection of the tooth surfaces [38, 67, 68]. Various stimuli can increase the salivary flow [69]. However, it is thought that some of the characteristics of saliva are dependent on variations among individuals.

Erosive lesions in enamel of deciduous teeth manifest more rapidly than on permanent teeth [70]. Apart from different morphology of the deciduous teeth, the lower salivary flow rate in children may be considered as one of the contributing factors. Decreased salivary flow rate has also been identified in some patients with eating disorders [71], and it has been proposed to use salivary flow rate as an indicator of dental erosions progression [72]. A study among a Finnish adult population revealed that low unstimulated salivary flow rate was a risk for dental erosive wear [67]. In addition, the asthmatic patients have a higher degree of dental erosion compared to non-asthmatic individuals due to diminished salivary flow most probably caused by asthmatic medications [73].

It has been suggested that the buffering capacity of saliva is of greater importance in cases of erosions compared to that of dental caries [74]. Weakened buffering capacity plays the role in higher occurrence of dental erosions in patients with gastroesophageal reflux disease (GERD) [75]. The most important buffer of saliva are hydrogen carbonate ions, and it has been shown that its concentration rises up to twelve times in stimulated saliva compared with unstimulated [68]. Other significant ions in saliva are dihydrogen phosphate (H_2PO_4^-) and calcium (Ca^{2+}), which have a role in remineralization [68], and fluoride (F^-), which enhance the same process [76].

Saliva has also an important role in the formation of acquired dental pellicle, the natural coat of the tooth surface. Studies have revealed differences in the composition of pellicle proteins between individuals with and without erosions [77, 78]. The pellicle that saliva forms on teeth varies in thickness both between individuals and between different locations in the mouth. Additionally, the pellicle, depending on its thickness, offers, to some degree, protection from acidic challenge [79]. However, when a new pellicle forms on an eroded surface it will delay remineralization. The capacity of a pellicle to protect against erosion was shown to be rather limited during a weak acidic challenge on enamel, whereas the effect on erosive challenge of dentine is insignificant [80].

Sjögren's syndrome is an autoimmune systemic disease followed by dry mouth [81]. Despite good oral hygiene, in some patient with Sjögren's severe dental erosions have been detected and it was suspected that it was mostly due to the poor quality of saliva [82]. For dental practitioners and medical doctors, it is demanding to collaborate in those cases, as dentists can often be the ones that first identify the signs of this disease. Patients should be given advices and instructions of the proper oral hygiene. They should be educated and counseled to follow the preventive measures in order to intercept the possible adverse effects of the disease to the oral health.

Enamel

The susceptibility to dental erosion may be a result of one or more environmental, phenotypic and/or genetic factors. Although it is known that individuals that frequently expose their teeth to acid are at higher risk for developing dental erosions, the knowledge about the molecular mechanisms underlying individual susceptibility to dental erosions is still elusive. The high prevalence of erosions among certain groups, and the studies demonstrating that not all individuals appearing to be at risk actually develop erosive lesions [5, 83], has influenced

research towards identifying genetic factors and excitations. So far, the findings display largely varying results and successes. This demonstrates the challenges of developing a study design with satisfactory sample sizes and durable phenotype definitions that allow adequate statistical power to identify genetic contributors. Even though the levels and frequency of acid exposure are difficult to control and determine, a clinical investigation by Søvik and colleagues demonstrated a connection between genetic variation in enamel formation genes and severity of dental erosions [7]. In this study, a significant association between tooth erosion and expression of enamelin and amelogenin, both being involved in mineralization of enamel, was demonstrated. On the other hand, Uhlen and collaborators found no evidence of an association between dental erosion and ameloblastin and several markers of tuftelin 1 [84].

Physical Activities

The awareness of physical health has increased among people. It is important for many to have a healthy lifestyle, which includes healthy diet and training. Often, during exercising, the secretion of saliva can decrease, due to dehydration and accelerated breathing with an open mouth [12, 85]. If sports drinks are consumed during that time, this creates favorable conditions for erosive processes to emerge. Previously, a relationship between consumption of sports drinks and dental erosive wear has been investigated, with positive results [38, 40, 42, 86]. Additionally, the typical healthy diet often comprises intake of unprocessed food, i.e. various smoothies and berries, which also are acidic [87].

Gastroesophageal Reflux (Disease) (GER(D)) and Eating Disorders

Gastroesophageal reflux (disease) GER(D) is a pathological condition with clinically observable symptoms. Exaggerated number of episodes of regurgitation of gastric acid into the oral cavity eventually can cause damage of the tissue due to acid exposure with very low pH of

0.8-2 [88]. In some patients, this repetitive reflux is often unobservable, and is known as silent reflux [89]. Several studies have found a relationship between GER(D) and dental erosions [90, 91]. Therefore, a collaboration between dental and medical practitioner is highly recommended in such case. Ruminations are a rare condition that is characterized by regurgitation and re-swallowing of the food. This is often associated with severe erosions, as the teeth are frequently exposed to gastric juice regurgitation, right after the food is being swallowed [92].

Individuals with eating disorders, such as anorexia and bulimia nervosa, practice varying degree of self-induced vomiting and binge eating. The overall risk for dental erosion is considered to be high in those patients [4, 93], mostly because of the frequent exposure of the teeth to both the acidic diet and the acidic content of the stomach. Data from Norway collected among 1960 adolescent aged 14-15 years has shown that eating disorders were present in 12.5% of the population [94].

Other Risk Factors

Some additional factors, such as use of medications, oral hygiene habits, occupational factors, and smoking influence the development of dental erosion. Certain medications have the potential to alter the salivary characteristics [95]. Medicines with low pH that are consumed frequently, especially those chewable or syrups might increase the susceptibility to dental erosions [96].

Proper oral hygiene is an important factor for the individual's oral health. There have been evidences of wear of the dentition in cases of vigorous tooth brushing, a case related to obsessive-compulsive disorders [97]. Tooth brushing during an acid challenge contributes to the wear [98]. However, the brushing action should not be postponed for a long time, as it provides a significant source of fluorides to the dentition, which is important for caries protection [12].

Some professions are frequently exposed to acids from the environment. For example, industrial workers are exposed to acidic fume or aerosols, professional swimmers are often exposed to pool water with low pH, professional wine tasters to acids from the wine. A review about occupational dental erosions comprising data from PubMed, Medline and EMBASE concluded that workers in battery, galvanizing industry and associated workers are in the group with the highest risk [99]. A research from 2013 investigating the prevalence of dental erosions in competitive and recreational swimmers, both exposed to gas-chlorinated swimming pool water, found erosions in 26% and 10% of the individuals from the competitive and recreational group, respectively [100]. An investigation from Norway among wine tasters confirmed the assumption about the occupational risk for dental erosion revealing meaningfully higher prevalence of erosions among wine tasters compared to the control group [5]. Hence, all these findings signify the importance of oral health and care promotion among these individuals. Regular examinations and monitoring, prophylactic treatment, wearing proper protective equipment while performing the work, advices about diet and oral hygiene should be strategically planned and applied.

It is also important to be aware of the fact that exaggerated intake of alcoholic drinks is associated with a high prevalence of erosion because of the frequent vomiting or alcohol-induced gastroesophageal reflux [101].

Diagnosis

Identification of dental erosions, classification and treatment may represent a challenge for dental practitioners [102], since signs and symptoms of the condition may be vague. The clinical appearance of the tooth surfaces is the most reliable feature for dental professionals to detect this condition. A recent study from Germany showed that dentists mostly use data such as lesion

depth, the size of the affected area and the presence of pain as diagnostic criteria [103]. A survey among Norwegian dentists indicated an overall positive response when it comes to education of diagnosis and treatment. However, regarding salivary analyses, classification and records of the erosive lesions, a lower positive response was obtained [104]. A proper diagnosis is particularly important in the early stage of dental erosion. The typical signs pointing at the presence of dental erosions, systematized by Lussi and Jaeggi, comprise a smooth, silky, dull enamel surface with the absence of perikymata, and intact enamel along the gingival margin [105]. When the erosive wear progresses, morphological changes become more visible, often present as concavities in enamel characteristically larger in its width compared to depth. Further progression disturbs tooth morphology even more. Nevertheless, it is important to distinguish erosive lesions from physiological dental attrition and dental abrasion.

Attrition and abrasion are both types of mechanical loss of tooth substance. The former is caused by tooth to tooth contact, whereas the latter is caused by contact of the tooth with another object, other than the tooth [9]. The erosion, attrition and abrasion often develop simultaneously in the mouth [106]. The clinical examination should be followed by a systematic anamnesis with respect to general health, diet, use of medications, and other risk factors. Individuals with erosions are often not aware of this condition. Therefore, a comprehensive investigation is needed for the risk factors to be revealed. Once identified, it is important with a follow up of the patient with erosions. Records, photographs, or study models of the dentition should be saved in order to document and track the progression of the lesions. In addition, the salivary flow rate and buffering capacity should be examined. Furthermore, dental erosive wear has to be distinguished from wedge-shaped defects. An inappropriate technique of tooth brushing may cause gingival recession and the exposure of cervical tooth surface with wedge-shaped defects [107]. Erosions are predominantly caused by the action of acids and aided by abrasion or attrition, while wedge-shaped defects are primarily caused by abrasion and occlusal

stress during inter-occlusal activities helped by erosive softening of the tooth. Wedge shaped defects are located at or apical to the enamel-cementum junction.

Eccles in 1979, and Smith and Knight in 1984 were the first who published and proposed scoring systems for the clinical diagnosis of dental erosion [108, 109]. Most scoring systems developed thereafter are modifications and combinations of these two. In 2008, the Basic Erosive Wear Examination (BEWE) scoring system was established by Bartlett et al [110]. It is based on a quantification of the size of the lesion as an estimated proportion of the affected surface [110]. Recordings of erosions in many studies imply the use of index teeth or surfaces where dental erosive wear is commonly present. It has been concluded that predictive locations for erosive lesions are occlusal surfaces of the molars, and palatal and buccal surfaces of the upper front teeth. The Visual Erosion Dental Examination (VEDE) scoring system can use all surfaces of all teeth (except for the incisal surfaces) as references. The system has been investigated and validated by Mulic and colleagues. [111]. This system differentiates not only between the size of the lesions but also between the severity of the lesions.

One must bear in mind that all scores should be interpreted with a dose of caution, since it is impossible to comprise all validity and diagnostic criteria in one single scoring system.

Treatment

In order to reduce incidence and progression of dental erosive wear prevention is of the essence. Early identification of risk factors for the development and progression of the condition and efforts to minimize those factors are crucial. Additionally, after identifying patients with erosions, dental practitioner needs to ascertain about the progress of the condition. When it progresses, monitoring is necessary to provide enough information about the rate of progression. Orthodontic study models, intraoral photographs, and use of indices or

colorimetric procedures are the main methods of monitoring [112, 113]. Furthermore, existing restorations may be utilized as reference in monitoring the progression.

Strengthening the dental hard tissues is the main purpose of the non-operative treatment of dental erosions. All patients should follow the ordinary recommendations to use fluoride toothpaste and/or fluoride lozenges/fluoride mouth rinse. There is consensus that there is a lack of evidence from clinical data for the effect of conventional fluorides (sodium fluoride and amine fluoride) to inhibit and prevent progression of dental erosions [114]. However, products with stannous fluoride and stannous chloride are shown to be effective in reducing the progression of this condition [115, 116].

Erosions restricted only to enamel seldom require restorative treatment. However, it is possible to apply flow composite or glassionomercement on the affected surface in order to reduce hypersensitivity [117]. The most common indications for restorative treatment of patients with severe dental erosive wear are functional and esthetical impairment, as well as sensations of pain. Even though there is no standard treatment of the dental erosions, the modern approach of minimally invasive techniques should be considered first [12]. However, even these can be quite complicated if a high number of teeth are affected by severe tissue loss, accompanied by remodeling of the alveolar bone, which results in loss of space for restorations.

In the case of local dental erosive wear in need of restorative treatment, restoration is often performed by composite or in some cases facets. On the other hand, in cases where the erosive wear is severely into dentine and affects a high number of teeth of the upper and lower jaw, the restorative procedures require more attention. There is often a need for reorganization of the occlusion, changes in the occlusal plane and vertical dimension, and full mouth rehabilitation. Minimal invasive direct techniques with composite are the first choice of treatment, but in some cases there are indications for ceramic or metal bound ceramic restorations.

Dental Enamel

Formation and Structure

Dental enamel, the hardest tissue in mammals covering the human tooth crown, exhibits several specific properties making it a unique tissue with highly organized and tightly packed crystallites. Once the tooth erupts into the oral cavity, the regenerative capability of enamel is fundamentally limited due to a loss of dental epithelium during eruption [118, 119].

All hard tissues in the body like bone, cementum, dentine, and enamel grow in layers. The movement of enamel-producing cells, the ameloblasts, brings them from the enamel-dentine junction to the surface of the enamel. The path pursued by each individual ameloblast is traced out by the prisms, while the movement of the ameloblast layer as a whole is mirrored by the incremental lines of enamel, the Retzius lines [120]. These lines, therefore, indicate the position of the ameloblast layer and of the developing enamel surface at different points of time and may evidence physiological or pathological events affecting enamel formation. Amelogenesis is accomplished through a temporally restricted and highly regulated series of events that include development of a specific extracellular matrix, matrix processing, and controlling the microenvironment of the developing enamel tissue [121-123]. These processes are highly regulated at the molecular level, with amelogenesis ultimately involving thousands of genes and their products [124, 125]. Not unexpectedly, these processes involve several developmental and regulatory pathways that could lead to abnormal enamel development and pathology.

The enamel is a highly mineralized tissue [126]. Most of the non-mineral component of dental enamel is water, with protein comprising less than 1% of the total enamel weight. The mineral content of enamel is made up of highly organized, tightly packed hydroxyapatite crystallites that comprise 87% of its volume and 95% of its weight. The structural richness of

dental enamel is related to the spatial arrangement of the hydroxyapatite crystals. These are not arranged at random; in mammals, the crystals are organized into a pattern of prisms (rods) and interprismatic (interrod) substance. It is estimated that the number of rods in a tooth ranges from 5 million in the lower lateral incisor to 12 million in the upper first molar [126, 127]. In general, the prisms are rod-like entities running from the dentine to the enamel surface, while the interprismatic substance constitutes a continuum in between the prisms [120]. The basis for a distinction between prisms and interprismatic substance is their differently oriented hydroxyapatite crystals; in the prisms, the crystals are oriented with their long axis roughly parallel with the long axis of the prism, while in the interprismatic substance, the crystals tend to be oriented perpendicular to the incremental lines (Retzius lines) [120].

Mouse Dentition

The mouse and human teeth exhibit substantial homology in tooth development with several common underlying molecular networks [128]. However, the dentition of the mouse is different with a distinctive molar pattern, continuously growing incisors, and a lack of tooth replacement. Additionally, the dentition in mouse is highly reduced, exhibiting only one incisor, separated by a diastema region to three molars, in each quadrant [129]. Similarly, rodent and human enamel exhibits the same basic structural elements, prism and interprism, however, the spatial arrangement of the prisms, i.e. the prism pattern, is considerably different [130]. Other differences include the speed at which enamel formation occurs [131] and the incorporation of iron in the superficial enamel layer of rodent incisors [132].

The mouse dentition consists of mandibular molars (M1inf, M2inf, M3inf), maxillary molars (M1sup, M2sup, M3sup) and mandibular and maxillary incisors (Fig. 1). Mandibular molars exhibit two rows of cusps, lingual (L) and buccal (B), whereas maxillary molars exhibit

three rows of cusps, lingual (L), buccal (B) and central (Fig. 1e, f). The rows of cusps are separated by groves of different depth. The teeth are not completely covered with enamel. At the tip of the cusps, obliquely oriented enamel-free areas are observed. These areas are facing distally in maxillary molars, and mesially in the mandibular molars, excluding the cusps B1 and L1 in first mandibular molar where areas are facing distally. The cusps of mandibular molars are tilted mesially, except for the cusp B1 and L1 on the first molar, whereas the cusps of the maxillary molars are tilted distally [133]. Considering the whole thickness of the enamel, the mouse molar enamel can be divided into four layers: a thin inner prism-free layer, an inner enamel with prism decussation, an outer enamel layer with parallel prisms inclined incisally, and a thin superficial prism-free layer (Fig. 1g) [133]. Mouse incisors are covered with enamel only on the labial aspect of the tooth (Fig. 1a, c), extending more laterally than mesially. The enamel of maxillary and mandibular molars contains a distinct yellow-brown iron pigment, with maxillary incisors being more pigmented [134].

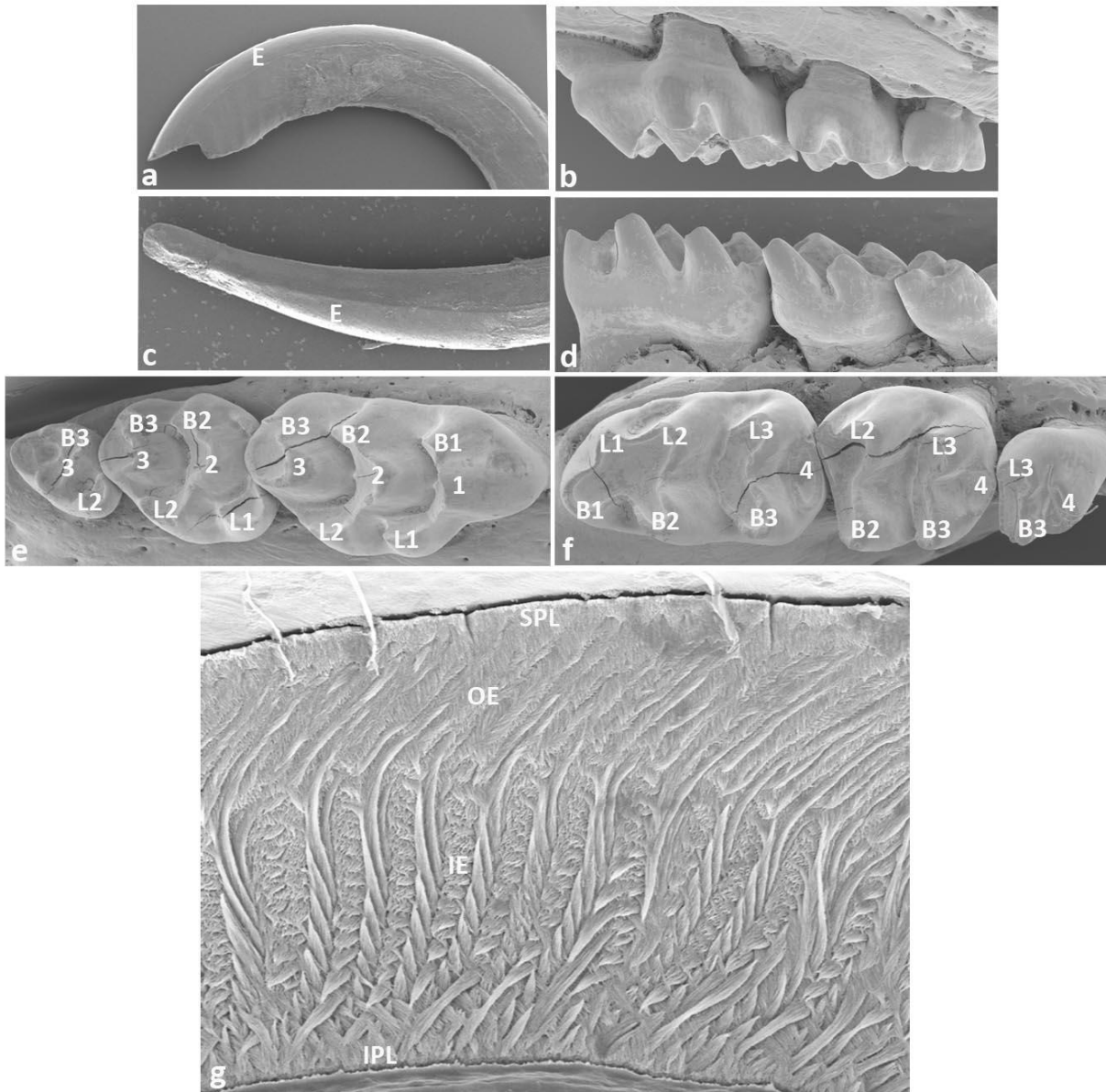


Figure 1. Mouse dentition – morphology and enamel structure

Maxillary mouse incisor (a) and lingual view of maxillary molar teeth (b). Mandibular mouse incisor (c) and lingual view of mandibular molar teeth (d). Occlusal view of maxillary (e) and mandibular (f) molar teeth showing the rows of cusps, lingual (L) and buccal (B). Mouse molar enamel (g) showing distinct four layers: a thin inner prism-free layer (IPL), an inner enamel with prism decussation (IE), an outer enamel layer with parallel prisms inclined incisally (OE), and a thin superficial prism-free layer (SPL). E = enamel.

Genetic Contribution and the Role of Aquaporins

Heritability is found to be an important contributory factor in many diseases. The developmental disorders of enamel and dentine and other disorders affecting the number of teeth, size, shape and color, are influenced by genetic factors. Genes are found to be associated with more than 40% of variance of caries in the population [135]. Additionally, a study investigating the association between enamel formation genes and dental erosive wear has found positive association in ENAM and AMELX [7]. Genetics could plausibly explain the variation among the level of dental erosions between people with similar exposure. Namely, it has been shown that some individuals do not develop erosions despite the acidic challenges to the dentition [4, 5, 84]. As a clinical association is not obvious in each particular individual, it has been suspected that the variations in genetic setup play its role.

Genome wide association studies (GWAS) are used to investigate the associations and linkage of deoxyribonucleotide (DNA) variants with known locations throughout the whole genome. Information provided from these studies serve as material for generating hypotheses. A recent GWAS among Finish adults revealed statistically significant evidence of several genetic markers feasibly associated with dental erosive wear [136]. On the other hand, candidate gene studies investigate previously created hypothesis regarding associations of genes and diseases. The common approach in genetic studies of oral conditions, specifically caries, is to detect a possible association between the single nucleotide variant (SNV) of a specific gene considering that its function is relevant to the disease development. SNV is the most common type of genetic marker. A SNV is a site on a chromosome, which varies at a single nucleotide. SNVs are common and there will be multiple SNVs within a gene, and hence they are commonly used in research.

In the past, it was assumed that water simply travels through biological membranes of the cells. However, rapid movements of water across some cells was still enigma. It had been anticipated that openings must exist in some cells which transfer large amounts of water. However, it was not until 1991 that the first aquaporin (AQP), the AQP1 was biophysically characterized [137]. Currently, the AQP family counts 13 members (AQP0-AQP12). It is now known that AQPs are expressed in cells of exocrine glands, gastrointestinal system, kidney, lung, eye and brain. They have also been found in some tissues, in which primary role is not water transport, such as red and white blood cells, adipocytes and skeletal muscles [138].

In salivary glands of rats and humans the AQP1 mRNA was detected and associated with myoepithelial cells, the contractile cells around the acini and small ducts [139-141]. In mice lacking AQP1, stimulated saliva secretion was different regarding the volume and composition [142]. However, in Sjögren's syndrome patients', biopsy of labial salivary glands revealed AQP1 decrease in myoepithelial cells, which could have a role in the pathogenesis of this condition [143]. AQP 2 has primarily been detected in kidney [144]. However, *AQP2* gene is clustered in the same chromosomal region as *AQP5* and *AQP6* [145]. AQP 3, 4, 6, 7 and 8 have been identified in salivary glands of humans and rats, but the level of presence and expression is still not clear [146-148]. AQP5 has a role in stimulated saliva secretion, where it affects the tonicity and viscosity [142]. In mice lacking AQP5, the membrane permeability of acinar cells was impaired to the great extent [149]. An *ex vivo* study testing association of *AQP5* variants and subclinical dental enamel loss revealed the association of some *AQP5* SNVs and enamel more resistant to demineralization [150]. Similar results showing the protective role of AQPs in the process of demineralization have been done previously [151, 152]. Such finding may serve as a guidance for future discoveries of the complex biological relationships of dental erosions pathology. AQP5 is expressed in the parotid gland, and contributes to the production of normal salivary flow [153]. A study revealed abnormal distribution of AQP5 in acinar cells

of salivary glands in Sjögren`s patients and suggested their influence to the diminished saliva secretion in this disease [154].

In wild type mice, the AQP5 distribution in salivary glands was restricted to the apical membrane of acinar cells whereas in non-obese diabetic (NOD) mice, which are generally considered as a good animal model for Sjögren's syndrome, [155, 156], it was rather presented at both basolateral and apical membrane [157]. These data are in concordance with the observations of loss of the ordered and polarized expression of AQP5 in human minor salivary glands and lacrimal glands in patients with Sjögren's syndrome [154, 158].

Aims of the Study

Overall aim:

The overall aim of the study was to gain further knowledge and a better understanding of risk indicators and the potential protective role of saliva for dental erosion. We aimed to investigate the risk indicators, i.e. some extrinsic factors and the influence of salivary factors for development of dental erosive lesions.

Specific aims:

- Paper I: To establish an animal model of extrinsic dental erosions where lesions of different severity can be created. To establish a standardized method with transversely ground mouse molars and observation in scanning electron microscope (SEM) that allows a registration of erosive lesions and lesion depths in small teeth like mouse molars.
- Paper II: To investigate the effects of experimental dental erosion in non-obese diabetic (NOD) mice with impaired salivary gland function and reduced salivary flow rate. To compare the observed effects with those observed in mice with normal salivary flow rate.
- Paper III: To explore the relationship between different dental erosive wear phenotypes in humans, aquaporins` genes, and selected environmental factors.

Methodological Considerations

Mouse Animal Models

Mice used for scientific purposes spend the major part of their lifetime in the laboratory environment. The nature of laboratory housing can significantly affect upon their welfare. The social behavioral patterns of wild mice obviously differ from those of laboratory mice. Mice are social animals that live in groups in the wild [159]. Social organization of wild mice is constantly changing due to the environmental conditions. However, in complex environments, such as in the laboratory, a higher density of mice is possible compared to in open areas. Mice exhibit thigmotaxis, i.e. their movements and orientation are responses to contact with other animals, and therefore they may not have a need for large housing areas [160]. However, every facility needs to meet certain requirements regarding the housing of the animals, such as air ventilation, airflow, light: dark cycle, temperature and humidity level. The mice in the present experiments were kept in individually ventilated cages. In these cages, the intra-cage air quality is high and accumulation of ammonia and carbon dioxide is reduced [161]. They were maintained on a 12-h light: dark cycle, at 21°C, with relative humidity of 65%.

Before the experimental erosive procedures, the mice were given water *ad libitum*. However, in order to exclude attrition of the teeth, both before and during the experiments, the standard laboratory fodder was softened prior to feeding. Fifty pieces of Teklad Global 18% Protein Rodent Diet (Envigo Teklad, Madison, WI, USA) were soaked with 165 ml cold tap water, sealed in a plastic bag, and left to soften overnight. Furthermore, the wire cages with solid bottom and bedding were prepared in order to reduce the wear of the dentition by attrition. Like all other rodents, mice as well have a habit to chew frequently [162]. The cages were carefully inspected before the mice were transferred into them. Any hard objects such as wooden sticks and plastic wheels were removed from the cages, and the animals were only

supplied with paper boxes and paper ribbons as a part of environment enrichment. The purpose of environmental enrichment is to provide the mice with options for activities and control over the surrounding [163]. Paper ribbons and paper boxes are a part of environment enrichment and mice use them to build nests or shelters. A study revealed that mice consumed more food and water in the absence of nesting material most likely due to boredom effect [164].

CD-1 mice, used in Paper I, are an easily available, inexpensive, vigorous animal model commonly used in research [165, 166]. They have efficient breeding results with large litter sizes and are quite simple to handle. In this study, 90 CD-1 female mice were randomly distributed into three experimental groups, which were merely offered two different acidic drinks, Red Bull sugar free sports drink (citric acid, pH=3.39) and Coca Cola drink (phosphoric acid, pH=2.27). In addition, a control group provided only with distilled water was included. Each group (n=30 animals), was further divided into triplicate subgroups, i.e. there was ten animals per cage. Previous similar animal studies have used between 64 and 100 animals [167-169]. The recent animal study on the effect of hypoxia on the formation of mouse incisor enamel [170] demonstrated well the individual variation in mice, where the effect of hypoxia varied considerably, among mice, among teeth in the same mouse, and among ameloblasts on the same tooth. In the present study we had three different groups (water, sports drink, and cola drink), and we also aimed to have three replicates for each group. Taken into the consideration the individual variation between mice as mentioned above, and the fact that it was not possible to monitor the drinking habits of each mice, only per cage, we found it reasonable to include 10 animals in each replicate. Furthermore, based on our group's long experience using mice for studies on dental enamel, we know that there are some technical issues dissecting and grinding the teeth of such a small size, compared to the rat teeth. Collectively, taken all this into the consideration, in this study we used a relatively high number of animals. The ethical aspect of this may be discussed; however, in order to establish a standardized animal model for further

studies of dental erosion, we considered it important to include many animals as a fundament for our measurements.

Non-obese diabetic (NOD) mice are mouse models characterized with salivary glandular hypofunction [171]. The very first signs of salivary gland impairment have been observed at an early stage of life, at 8 weeks [172, 173]. Currently, NOD mice are used in studies of Sjögren's syndrome, an autoimmune systemic disease with ocular and oral dryness [156, 174]. For the second study (Paper II) 42 phenotypical NOD/MrkTac female mice, about 9 weeks old, were purchased from Taconic Biosciences (Ejby, Denmark). NOD/MrkTac female mice from Taconic Biosciences are very expensive experimental animals. Initially, we ordered 48 animals and we aimed to have equal number (N=12) of animals in each group. However, at that time-point, it was not possible for Taconic Biosciences to create and ship to us more than 42 animals at the certain age that was appropriate for our study design. Therefore, we had to divide the number of animals that we received. Being aware of higher probability of illness in the experimental groups, we decided to include 11 mice in each experimental group and 9 mice in the control group, in addition to 9 wild type control mice. It turned out to be more death in the experimental groups, so we ended with more equal distribution of the survived animals. Prior to experimental use, the animals were kept in the facility for about 7 weeks for them to reach the age of 16 weeks, the time point at which they develop dysfunction of the salivary glands resulting in significantly reduced salivary flow rate [175, 176]. At the age of 16 weeks, the mice were randomly distributed into four groups, i.e. three experimental and one control group. Each of the experimental groups was equally provided with Red Bull sugar-free sports drink (citric acid, pH 3.39) (n = 11), Coca Cola light drink (phosphoric acid, pH 3.10) (n = 11), and Coca Cola drink (phosphoric acid, pH 2.27) (n = 11), respectively, while the control group was provided with distilled water (n = 9). In addition, we included a wild-type group, provided also with distilled water. Each group was further divided into triplicate subgroups.

Prior to the experiments in Paper I and II, the changes in pH of both the sports drink and cola/cola light drinks were monitored over three days, and the results showed no significant changes in pH. All the bottles were replaced three times per week, and the consumption of drinks in each cage was recorded in both studies. After the 6-week experimental period, the animals were sacrificed by cervical dislocation, and their heads were fixed in 70% ethanol. All animals were weighed at the start and at the end of the experiment.

Scanning Electron Microscopy

Microscopy is a useful tool in dental studies. In everyday practice, optical microscopy is used in dental clinics. In research, in order to obtain surface information, electron microscopy is widely used. Scanning electron microscopy (SEM) is an approach that makes significant contribution by allowing detailed three-dimensional visualization and providing information that goes beyond the data obtained with other instruments. SEM is primarily a method for studies of external morphology and crystalline structure [177] using different magnification sizes. Images are obtained by detecting secondary electrons, providing information about the surface topography scanned by the beam of primary electron. Therefore, it represents an excellent choice for the study of the dental enamel. Enamel is built of the mineral hydroxyapatite, packed in small crystals. These crystals are organized in a characteristic pattern, packed tightly in two different directions, prisms and inter-prism. In SEM, dental enamel can be analyzed either by studying the surface structure, or section of enamel, after the tooth has been grinded and etched at the certain point.

Prior to analyzing in SEM, the tooth specimen has to be prepared. Certain protocols must be followed while preparing the sample. Firstly, it is of utmost importance that the specimen is very well cleaned and dry. Only after this has been fulfilled, the specimen is ready

for the coating procedure. Furthermore, in case of grinding, etching, and embedding the sample, the appropriate resin needs to be chosen, as well as acid etching regime and the grinding technique. In our studies (Paper I and II), both left and right upper and lower jaws containing all three molars and incisor teeth were dissected out and afterward fixed in 70% ethanol. After fixation, residual soft tissue was carefully removed. This was performed under a light stereomicroscope. The specimens were cleaned under running tap water by gentle brushing movements and left to air-dry overnight. In the first work (Paper I), the samples were mounted on brass cylinders with cyanoacrylate glue, sputter-coated with 30 nm platinum and observed in a Philips XL30 ESEM (Philips, FEI, Netherlands) operated at 12 kV. For the purpose of the second project (Paper II), the samples were observed in GeminiSEM 300 SEM (Zeiss, Oberkochen, Germany), operated at 5 kV. Sputter coating with a metal layer is of utmost importance when analyzing the specimens in a microscope with focused electron beam. This is performed in order to provide the adequate conductivity and minimal obscuring of fine details. When observing in SEM we used the approximate acceleration voltages of 12 and 5 kV in Paper I and II, respectively. Increase in focus depth is dependent on working distance. This principle is important in analyses of objects with uneven surfaces. On the other hand, flat surface request a shorter working distance and better resolution.

After the morphological analyses of the whole tooth specimens were completed, the lower jaw segments containing all three molars were embedded in epoxy medium (Epon), and ground transversely. The grinding procedure was performed under a stereo-microscope using grits 800 and 1200 3 M waterproof silicon carbide paper (3 M, St. Paul, MN, USA) in a specially designed apparatus [178]. The ground surfaces were then polished by grinding the specimens against the backside of the 3 M waterproof silicon carbide paper with 0.05 μm particle size alumina powder (Buehler Micropolish, Buehler, Lake Bluff, IL, USA) in water. Additionally, the specimens were carefully brushed under running tap water in order to remove the residual

particles. Subsequently, the teeth were etched for 45 s in 1% nitric acid, air-dried overnight, sputter-coated with 30 nm platinum and observed in SEM. For the transversely ground molars the whole procedure (grinding, polishing, etching, air-drying, sputter-coating and observing in SEM) was repeated, creating two transversely ground planes for observation (Paper I), and three different planes (Paper II). Etching of the specimens with acid for the certain time period was performed in order to expose the structure of the enamel in the sectioned planes. Etching with the acid will always result in a structure-dependent surface topography of the enamel, independent of the orientation of the section due to the prism and inter-prism crystals being differently oriented [179]. The amount of the enamel that is removed by etching is directly proportional to the concentration of the acid and etching time. The specimens were etched with nitric acid, as nitric acid has good properties regarding minimal precipitates formation [180]. While immersing the specimen in the acid with a pair of tweezers, it was quite energetically moved throughout the acid in order to prevent reprecipitation of dissolved material. In case of extensive etching, interpretation of the structure might be challenging due to the improperly accentuated topography. The ionic strength of pH of the acid can diminish with time, and it is therefore desirable to frequently replace the solution. Specimens were rinsed under running tap water immediately after the etching procedure in order to halt the etching process. Even though most of the remaining moisture is removed during coating of the sample in the vacuum sputter chamber, it is desirable to remove as much as possible prior to this step. Therefore, the specimens were set to drying prior to the sputter coating. The time needed for the moisture to evaporate depends on the size of the specimen. Having in mind that mouse molars are quite small specimens, in the studies (Paper I and II) the samples were air-dried overnight at room temperature. When dried at higher temperature, i.e. in a drying cabinet, most of the samples exhibit crack formations on the tooth surface.

For the purpose of the first study (Paper I), two ground planes were created. The first plane (T1) was positioned on the mesial aspect of buccal cusp B2 and lingual cusp L2 where the tip of the cusps exhibits enamel-free areas [133]. The subsequent plane (T2) was ground further in distal direction ending on the distal aspect of buccal cusp B2 and lingual cusp L2 where the tip of the cusps is covered with enamel. The T1 and T2 planes were positioned in an area where the occurrence of dental erosions on the first molars were noted when the whole teeth were observed in the SEM. In the second study (Paper II), three ground planes were made. In addition to T1 and T2, as described above, the final third plane (T3) was positioned reaching the mesial aspect of the buccal cusp B3 and lingual cusp L3.

Human Population and Questionnaires

The study population in Paper III was based on the population of 795 dental patients from a previously published cross sectional study [21]. The material was obtained through a collaboration between the University of Oslo and Tannhelse Rogaland (Public Dental health region in Western Norway) in 2012, at five randomly selected Public Dental Service (PDS) clinics in Rogaland County. The collection of the material was done during recall examinations, and included three different aspects: a clinical examination, a questionnaire, and collection of a saliva sample. The patients were 16, 17, and 18 years of age, healthy and unrelated. Participants were clinically examined by calibrated dentists and dental hygienist (n=8; mean inter-examiner agreement (κ_w) = 0.55). The purpose of the examinations was to identify and grade erosive lesions.

As a referral system, VEDE was used, established by Mulic and colleagues [111]. This system is assigned five different scores based on the erosive wear severity, i.e. erosion free-score 0; initial enamel loss, no dentine exposed-score 1; pronounced enamel loss, no dentine

exposed-score 2; dentine exposure with less than 1/3 of the dentine surface exposed-score 3; 1/3-2/3 of dentine exposed-score 4; more than 2/3 dentine exposed-score 5. Representative (index) surfaces were the occlusal surface of the maxillary and mandibular first and second molar and the labial and palatal surface of the maxillary incisors and canines [111].

In case of more than three index surfaces with dental erosions, participants were classified as affected. Based on the erosion score, three dental erosive wear phenotypes were defined: A- unaffected individuals (control group), B -individuals with dental erosive wear affecting enamel only (score 1-2) (mild erosions), and C- individuals with dental erosive wear into dentine (score 3-5) (severe erosions). There are several considerations to validate the choice of the specific age group and study population. Firstly, the adolescents at this age might present an appropriate population, as other types of wear, such as abrasion and attrition, are here less prominent compared to in older individuals [181, 182]. Furthermore, studies have demonstrated high prevalence of erosive wear in adolescents [45, 183]. Additionally, by the Norwegian regulations, children and adolescents at the age of 18 years and younger, are financially covered by the government for all dental visits at PDS clinics and are routinely scheduled for dental examinations. This fact facilitates data collection to the great extent.

A self-administrated questionnaire was completed by the patients prior to the clinical examination. The questionnaire contained questions regarding the background, behavioral and acidic dietary variables. For the purpose of Paper III, only dietary and oral hygiene habits were included. In order to ensure the intelligibility and clarity of the questionnaire, it was tested by a pilot group prior to the study. Additionally, completing the questionnaire preceded the clinical examination, which made it possible for the adolescents to ask the clinicians in case they needed clarification of the questions. This is an important fact, as it can affect the positive response rate, hence limiting the risk of a non-response bias [184]. One must bear in mind that there is no standardized questionnaire for evaluation of risk indicators on dental erosive wear.

Therefore, the reliability of the data provided from the questionnaire must be considered with certain caution, as the answers are based on the participants' ability to recollection of past events, in addition to the possibility of selective reporting.

Saliva Sampling and DNA Extraction

A sample of unstimulated saliva was collected from 795 dental patients in a quiet, isolated room. Participants were guided through the collecting process and they were instructed to relax in an upright sitting position. After few minutes, a standardized collection of saliva was performed, by letting the saliva drip into a graduate plastic tube. The patients were asked not to exercise, or drink and eat an hour prior to the saliva collection. Thereafter, the samples of saliva were stored in Oragene DNA self-collection kits (DNA Genotek Inc. Ottawa, ON, CA) at room temperature. Genomic DNA was extracted according to the manufacturer's instructions. Genotyping of 38 SNVs related to immune response, enamel formation, and dental development was performed using the TaqMan chemistry (Applied Biosystems, Foster City, CA, USA). According to the aim of the present investigation, only nine SNVs were used.

Polymerase chain reaction (PCR) is a method of genetic analysis used broadly as it has an important role in quantitative DNA analysis. Real-time PCR allows the identification of the amount of initial DNA in the sample before the amplification by PCR. An advantage of real-time PCR is the capacity to monitor the progress of DNA amplification in real-time. This is achieved with instrumentation and chemistries. Chemistries commonly consist of special fluorescent dye and probes. Hydrolysis probes are sequence-specific dually fluorophore-labeled DNA oligonucleotides. TaqMan probes are commonly used [185]. One fluorophore is named the quencher and the other is the reporter. Hydrolysis probes have a high specificity, because only sequence-specific amplifications are measured. On the other hand, different sequences

require the synthesis of different probes. In order to define the DNA concentration and purity of our samples, the spectrophotometry (NanoDrop) was used. The DNA concentration was evaluated at 260 nm and the ratio of readings at 260 and 280 nm was used to estimate the DNA purity. The samples were afterwards diluted to 2 ng DNA/ μ l with TE buffer (10 m M TrisHCl, 1 m M EDTA, pH 8.0). The genetic variants (SNVs) were selected within candidate genes potentially involved in immunity and salivary contribution. The selection was based on allele frequency and linkage disequilibrium structure of the locus. Genotyping was performed using the TaqMan assay and PCR. The primers, probes and universal master mix were obtained from the Applied Biosystems 7900 HT Sequence detection system machine. Two TaqMan probes, used in these analyses for the allelic discrimination assay, consisted of an oligonucleotide with a 5' reporter dye and a 3' reporter dye. During PCR analysis, the forward and reverse primers hybridize to a specific sequence of the target DNA. The TaqMan probe hybridizes to a target sequence within the PCR product. A result of the separation of the reporter dyes from the quencher dye is the increasing fluorescence for each of the reporters. This increase is measured and is a direct consequence of target amplification during PCR. A quantitative PCR was performed in a total volume of 1.8 μ l (1.2 ng DNA/reaction, 0.6 μ l TaqMan PCR master mix + SNV assay) (Applied Biosystems). The thermal cycle was carried out by starting with a hold cycle at 95°C for 10 min, followed by 40 amplification cycles at 92°C for 15 s and at 60 ° C for 1 min.

Measurements and Statistical Analyses

In Paper I and II, SEM images of the transversely ground and etched plane T1 were used for measurements of tooth height and enamel thickness in control and experimental mandibular first molars. Mean values and standard deviations were calculated using Microsoft Excel

Worksheet. The step initiation at the eroded lingual enamel in experimental molars, was calculated by measuring the distance between the horizontal line at the level of the step and the corresponding line at the level of the enamel-cementum junction. Measurement data were tabulated and analyzed using the Statistical Package for Social Sciences 22.0 for Windows (SPSS Inc., Chicago, Illinois, USA). One-way analysis of variance (ANOVA) followed by the Tukey post-hoc test, and independent t-tests were used for the evaluation of data. P-values < 0.05 were considered statistically significant. When considering the accuracy of the measurements, it is likely to assume that there is some variation in the tooth size between the mice, and that our measurements may have been influenced due to these variations. However, previous studies from our laboratory have shown that these are minor in mice where the body weight is not significantly different [186-188]. Therefore, using CD-1 strain in the Paper I where the morphology and size of the mouse molars have previously been thoroughly described [133], and including water as a control, we feel that this may not have affected the accuracy of the measurements significantly. The loss of enamel layers on the lingual aspect of the tooth is best observed in transversely ground planes through the tooth. Achieving ideal transversal ground plane of mouse molars through the correct cusps is technically difficult due to their small size, and consequently, the position of transversal sections may have varied slightly. However, it was considered unlikely that this slight variation would mask the significant differences in enamel thickness between the groups.

For the purpose of Paper III, the statistical analyses were performed using the PLINK software [189]. Allele frequencies as well as Hardy Weinberg equilibrium were calculated. Genotyping and haplotype analysis of the nine *AQP* markers were performed in order to compare the affected and unaffected individuals and to determine overrepresentation of the studied alleles. Thereafter, an association analysis was performed to compare patients' genotypes and phenotypes and look for an association between chosen SNVs and dental erosive

wear experience. The data collected previously through the questionnaires were included as covariates in the logistic regression analysis with the purpose to investigate the association between selected nine *AQP* markers, dental erosive wear phenotypes, dietary preferences and oral hygiene habits. Each covariate was run separately. The *p* values below 0.0011 were considered statistically significant (0.05/45, the denominator is the number of genetic markers tested), after inducing the Bonferroni correction. The *post hoc* sample size was calculated based on comparing erosion prevalence in two groups. It was assuming that the two groups were of equal sizes. Additionally, in order to have 80% test power to detect a difference of at least 10 percentage points between the groups, a minimum of 610 participants had to be included. There were 705 participants in this study, which indicated that the study had sufficient test power.

Ethical Considerations

The experimental animals used in this research (Paper I and Paper II) were kept in accordance with Norwegian regulation and legislation (Norwegian Regulation on Animal Experimentation of 2015 based on the EU directive on the Protection of Animals used for Scientific Purposes 2010/63/EU and the Norwegian Animal Welfare Act of 2009). The experiments were approved by the Norwegian Food Safety Authority FOTS IDs, 12710 (Paper I) and 16721 (Paper II). The ethical aspect of giving acidic and high-sugar soft-drinks to NOD mice prone to develop diabetes is important. Although only one drink, i.e. cola drink, contained sugar, considering guiding principles underpinning the humane use of animals in scientific research, the three Rs, it was not reasonable to include more than about 10 NOD mice, prone to develop diabetes, in each group.

For the study including human saliva samples and clinical data (Paper III), approval was obtained from the local Regional Committee for Medical Research Ethics and the Norwegian

Social Science Data Services (2011/1602/REK). This protocol was additionally approved by the University of Pittsburgh Institutional Review Board (PRO12110620). All the participants gave their written and informed consent prior to the study onset.

Summary of Results

Paper I

The molars of the control mice, and the incisors of both control and experimental mice, were unaffected. However, the experimental molars exhibited various degrees of erosion with a specific erosion pattern in both sports drink and cola drink molars. The lingual surface of the mandibular molars was most eroded, and a distinct step representing the border between the unaffected cervical and affected occlusal part of the teeth, was observed on the lingual aspect of all the mandibular molars from the experimental groups. The presented step was evident in the sports drink molars at about 274 μm , and at about 183 μm in the cola drink molars, as measured from the enamel-cementum junction. Going from this step in an occlusal direction, the enamel was gradually lost, with complete loss of superficial enamel and partial loss of outer enamel in sports drink molars. However, a complete loss of superficial and outer enamel, and gradual loss of inner enamel was observed in cola drink molars. At this level, dentine erosion was also observed, as judged by the lingual outline of the dentine surface.

The cola drink exhibited higher erosive effect on mandibular molars compared to sports drink. On the occlusal aspect of the teeth, erosion had increased the size of the enamel-free areas, which were continuous with the lingual dentine. The lingual tooth height, compared to control, was about 34% and 18% lower in the cola drink and sports drink molars, respectively. Compared to the control molars, the lingual enamel was about 23% thinner in the sports drink molars and totally eroded on the certain lingual areas of the cola drink molars. However, the enamel on the buccal aspect of the teeth was unaffected. Both lingual and buccal cusps were rounder and reduced in height compared to controls. In the maxillary molars, a distinct erosion step on the lingual side was observed only on the first molar in the sports drink group, and on first and second molar in the cola drink group.

Paper II

The NOD mice, exhibiting low salivary flow rate at a certain age, served as a beneficial model for studying reduced saliva as a risk factor for dental erosive wear, however, the frequent development of diabetes and resulting death was challenging. One NOD mouse from the cola light group was found dead during the experiment, whereas 14 mice were terminated during the experimental period due to observed abnormal behavior, sickness, lethargy, and poor general health and appearance. Reduced salivary flow, together with a high consumption of acidic drinks, resulted in severe erosion of NOD mice molars. In all experimental NOD mice groups, acidic drinks exhibited higher erosive potential compared to corresponding groups in the mice with normal salivary flow. Mandibular molars were considerably more eroded than maxillary molars. Erosive lesions were evident in increased succession from sports drink, cola light to cola drink exposed mandibular molars, with the lingual tooth height being about 23%, 26%, and 37% lower, respectively, compared to the control. The lingual enamel was about 48% thinner in sports drink molars and 62% thinner in cola light molars. In cola drink molars, the lingual enamel was totally eroded, and significant erosion of dentine was evident.

SEM observations of the morphology and transversal sections of mandibular first molars from both control and all three experimental groups, demonstrated that the erosive process started at the top of the cusps and subsequently extended in the cervical, mesio-distal, and pulpal direction. A loss of enamel was initiated at the occlusal half of the tooth, including the tip of the cusps, resulting in reduced enamel thickness and dentine exposure at the tip of the cusps L1 and L2. The persistent erosion resulted in further loss of dental enamel on cusps L1 and L2 in both the cervical and mesio-distal directions, and simultaneously with the evident initiation of enamel erosion at cusp L3. At this point, synchronous loss of enamel towards the underlying dentine together with further progression of the erosive step in cervical direction, was observed.

Paper III

Of total 795 adolescents, 90 individuals were excluded due to unavailable data (lost during the genotyping procedure). Therefore, the final sample consisted of 705 individuals, 342 males and 363 females. Of 705 individuals, 39.6% (n=279) were defined as having no dental erosive wear, 44.3% (n=312) with only enamel affected by dental erosive wear, and 16.1% (n=114) had dental erosive wear both in enamel and dentine.

Associations between the tested SNVs and dental erosion phenotypes were examined first. The individuals without dental erosive wear represented the comparison group, while individuals who exhibited mild (VEDE score 1-2) and severe (VEDE score 3-5) dental erosive wear comprised the affected groups. The results did not show any affirmative association. Thereafter, the individuals without dental erosive wear were excluded. Patients with mild erosive wear now represented the comparison group and the severe erosive wear individuals the affected group. Associations were found with rs2878771 (*AQP2*) in the genotypic (p=0.02) and dominant (p=0.03) models, as well as with rs3736309 (*AQP5*) in the allelic model (p=0.02). Subsequently, logistic regression analyses including covariates from the questionnaire were performed for the same groups as cited above and several significant associations were present when covariates such as frequency and amount of different acidic drinks consumption, daily brushing frequency and frequency of acidic sweets consumption were included. The most significant results were found between two SNVs in *AQP2* (rs2878771 and rs3741559) and soda intake frequency (p values equals to 0.00051 and 0.00096, respectively) and between *AQP5* (rs3736309) and wine amount (p=0.0008). When asked about soda intake frequency and wine amount, 89% and 87% of the participants reported high scores (3, 4, or 5) respectively. Additionally, the association between rs467323 (*AQP2*) and erosive tooth wear was dominated by females, who appear to be more likely affected.

General Discussion

The overall aim of the present work was to further investigate risk factors for dental erosive wear, and to study the development and progression of the erosive lesions. In Paper I, we explored the influence of extrinsic acid sources as a risk factor for development of dental erosions in a mice model. The model developed in this study, including an experimental procedure with SEM, provided us the opportunity to design the subsequent study (Paper II), which highlighted and investigated the salivary contribution to the development of dental erosive wear, since saliva is assumed to play a very important role in protection against this condition. Therefore, mice with impaired salivary gland function were exposed to acidic drinks for a certain period. The findings obtained in this study indicated the significance of salivary contribution to the development of dental erosions. Thereafter, in Paper III, we aimed to expand the area of interest, investigating more profoundly the salivary contribution to the dental erosive wear development. For that reason, saliva samples from human subjects were analysed in order to study the relationship between salivary components and dental erosive wear phenotypes.

The estimated prevalence of dental erosion is high, mainly among adolescents [20, 190, 191]; yet, pathological mechanisms are not entirely understood. Therefore there is a high need for a standardized model of extrinsic dental erosion that may serve as a reference for further studies [192]. The novel *in vivo* animal study presented in this thesis provided a unique opportunity for a more detailed insight into the risk indicators, process of development and progress of erosive wear, with the foremost ambition to contribute to creation and implementation of forward-thinking preventive strategies. The possibility of controlling the experimental conditions to some extent is an advantage of animal model. Moreover, invasive experimental procedures in humans, which result in permanent loss of hard dental tissue, are highly unethical. The current approaches suggest the genetic component underlying

environmental factors, and the balance between them, as fundamentals of etiology [2]. However, the susceptibility to erosions varies considerably among individuals [5, 6, 193]. Therefore, additional accent has been put on investigations of the individual's genetic predisposition to dental erosive wear [6, 7, 136]. The affirmative findings from previous studies have raised some novel questions of important genetic markers involved in the pathology of erosive tooth wear. It is plausible that genes regulate the enamel resistance to erosion, the salivary composition and flow, the behavioral patterns and the immune response. Understanding the basics of those complex relations opens doors for further associations yet to be explored.

Frequent exposure of the dental tissues to acidic food such as carbonated drinks, acid snacks, sour candies and natural acidic fruits juice is linked to dental erosions [42, 194]. The most common acids from the beverages, such as citric acid, phosphoric acid, carbonic acid, ascorbic acid, and malic acid, are considered to have an erosive effect [195, 196]. In Paper I, the animal model of dental erosion was established as a model for studying tooth surface loss caused by acidic drinks. Thereby, the impact of acidic drinks on dental tissues was also investigated. As far as we know, this was the first study where the morphology of the eroded tissues was analyzed into detail and thereafter comprehensively described. The Paper II aimed to investigate the influence of saliva by exposing the teeth of the mice with impaired salivary gland function to the acidic drinks. As the intention was to explore the effect of reduced salivary flow rate on development of erosive lesions, a similar experimental condition as in Paper I were designed in order to allow the comprehensive comparisons of the results. NOD mice develop a substantial salivary gland impairment characterized by presence of inflammatory cells in the salivary gland tissue and reduced salivary flow rate, between week 17 and 24 [175, 176]. By having this in mind, it was not possible to use animals of the same age as in Paper I, where the study was initiated at 7 weeks of age. Therefore, in Paper II, the experiments were initiated in 16 weeks old NOD mice.

Greatest erosive impact was observed on the lingual surface of mouse mandibular molars, exhibiting no significant differences between the first and the other mandibular molars (Paper I). The integrity of the maxillary molars was less impaired compared to the mandibular teeth, and here the first molars were more affected compared to the second and third molars. These findings are in accordance with clinical findings [26]. The plausible explanation for this would be anatomical relations where the acid is present for a longer time in the sublingual compared to palatal area of the oral cavity. On the other hand, the buccal enamel of the mandibular cola drink molars, which exhibited severe erosive defects lingually and occlusally, was not affected. It is, however, likely to think that the acid remained in the muco-buccal fold long enough time to induce some erosion on the buccal aspect of the molars. In human subjects, the clinical situation can vary, as the erosive wear occurs on the buccal aspect of a molar more often than lingually [197].

The systematic analyses of the mice teeth revealed a specific erosion pattern, with a small individual variation in the erosive effects between the mice within the same experimental group. It was hypothesized that this variation may be a consequence of variable drinking habits and the amount of drink consumed by a single animal, which was not possible to control, or due to other individual factors, such as salivary flow and saliva buffering function. However, the overall consumption of drinks was not significantly different between the groups. The results from the study using NOD mice as an animal model indicated that the lingual and partially occlusal surface of mandibular molars were extensively eroded after the exposure to acidic drinks. These findings supported the earlier identified erosion pattern in rats [167-169], and our findings obtained in Paper I. In general, the results demonstrated that the acidic drinks resulted in higher erosive tooth destruction of NOD molars compared with that in CD-1 mice with normal salivary flow (Paper I).

As anticipated, the incisors of both the CD-1 and NOD mice were not affected by erosive wear. Mouse incisors are continuously growing teeth with the enamel covering only the labial aspect of the tooth [170]. It is reasonable to assume that the erupted part of the incisors was not in contact with a considerable amount of acid for a long enough time. Moreover, the enamel of the mouse incisors contains recognizable yellow-brown iron pigmentation, which is found to be more resistant to acid [134].

Further, in both Paper I and II, the erosive effect on the enamel layers in different experimental groups was explored into details. This was performed by studying the loss of enamel layers (Fig. 1) using the SEM. On the lingual aspect of the molars exposed for acidic drinks, an obvious border between the affected occlusal part, and the unaffected cervical part of the molars was observed. However, even in the cases of complete erosion of enamel above this characteristic step, in cola drink and cola light drink mice, the cervical part of the enamel remained unaffected. It is possible that this is due to the gingival coverage of the enamel in this region, which could prevent the contact of acid with the dental tissue. Nevertheless, the presence of such an abrupt transitional step remains unclear. However, the same phenomenon was also observed in humans with a typical cervical edge of unaffected enamel due to gingival coverage in the region [107]. Erosive lesions were evident in increasing succession from sports drink, to cola drink (Paper I), and from sports drink, to cola light, to cola drink molars (Paper II). However, the experimental NOD mice groups exhibited higher degree of erosions compared to corresponding groups in the CD-1 mice. Additionally, compared to the control in NOD mice, the lingual tooth height of first mandibular molars was about 23% and 37% lower in sports drink and cola drink mice, respectively. The reduction of lingual tooth height in CD-1 mice was about 18% and 34% in the sports drink and cola drink molars, respectively. An even more dramatic outcome in the experimental NOD mice was observed in enamel thickness with about 48% reduction in the sports drink molars and total destruction of the enamel including

significant erosion of dentine in the cola drink molars. It was also evident that the erosive step in the experimental NOD molars was extended significantly further in the cervical direction compared with that in the mice with the normal salivary function. However, most of the cervical part of the lingual enamel in the experimental NOD mice was not affected. It is proposed that, when the erosion reaches the enamel at the gingival margin, the destruction is no longer occurring from the superficial enamel layer and into deeper enamel layers, but rather in the vertical direction. There is a possibility that the same pattern may be found in human enamel [105].

Often, it is difficult to distinguish the actual cause of dental tissue loss in such exaggerated conditions where erosion occurs simultaneously with attrition and abrasion [106]. Laboratory mice are provided with commercial pellets, which have an abrasive effect and increase tooth wear when compared with powdered diet. A study investigating the influence of the rough pellet food on rat teeth found severe attrition on the lingual surface of the first mandibular molar compared with the molars of the rats that consumed powdered diet only [167]. Additionally, erosive sports drink combined with rough pellet food caused greatest tooth surface loss [168]. In order to minimize the attrition, all the mice in our studies were provided with standard laboratory fodder previously softened with water during the whole experimental procedure. Specifically, in the study using NOD mice (Paper II), softened standard laboratory fodder was served to mice as the only food from the time point when they arrived at the animal facility until the beginning of the experiments, and continuously during the 6-week long experiment. Since all molars in mice reach the occlusion at the age of 35 days [133] it is possible that some of the occlusal wear occurred before the mice were provided exclusively with the softened food. Of course, a certain level of wear may have occurred during the experiment as well. Even so, the enamel loss observed on the lingual part of the sports drink and cola drink molars, is mostly due to erosion. The significant finding in the study including NOD mice

(Paper II), judged by the tooth morphology at the end of the experiment, is that the mandibular molars in the control group did not show increased tooth wear compared to that in the control CD-1 mice (Paper II). Therefore, we may conclude that the tooth wear observed in the experimental NOD molars was mainly due to erosion, and that these results were comparable with findings in CD-1 mice (Paper I).

In our animal studies the mice were exposed to acidic drinks continuously for six weeks. These exaggerative conditions might have contributed to the rapid destruction of the tooth surface, and they may not entirely reflect the human exposure. In humans, the consumption of acidic drinks is more varied and often combined with other drinks. Due to this circumstance, the results should be reasonably extrapolated to humans. Nevertheless, these findings certainly raise questions of the possible relation between the acidic exposure and dental tissue destruction.

The experimental design in Paper I comprised a sugar free sports drink (Red Bull) and the other containing sugar, cola drink (Coca Cola), whereas in Paper II, cola light drink (Coca Cola light) was also included. Even though the exposure to sugar from the drinks could possibly have influenced on the outcome, our results did not show any caries lesions in the molars of the cola drink mice. The process of chemical dissolution of dental tissues comprises numerous factors. For the most, it depends on type of acid, and physical and chemical factors of the acidic solution [196]. Citric acid has a higher titratable acidity compared to phosphoric acid, in addition to the ability of chelating calcium ions. Therefore, it is considered more erosive towards the dentition than phosphoric acid. Chelation represents the ability of some ions, such as citrate, to form a complex with calcium. This increases the degree of undersaturation and thereby favoring demineralization [198]. Bearing this in mind, it was assumed that the sports drink would be more erosive than cola drinks. However, the results of the present studies displayed the contrasting findings with cola and cola light drink causing more erosions in the

dental tissue. A study from 2016 showed that the chelation process does not have a significant effect on enamel erosion under flowing conditions and that the dissolution by citric acid depends on more than a single factor [199]. Consequently, we assume that the low pH of cola and cola light drinks had a significant role in the development of dental erosions.

In the animal studies, two and three ground planes of the mouse molars were made, respectively, in order to measure the tooth substance loss. The grinding procedures were performed in a previously designed apparatus [178]. However, due to the small size of mice teeth, achieving an ideal transversal ground plane through the correct cusps is technically difficult. This could potentially affect the accurateness of the measurements. Nevertheless, the differences in enamel loss between the groups were significant, which indicates that these small variations could not affect the overall conclusions. Furthermore, as the presented experimental groups displayed different degrees of erosion, it was possible to study the pattern and consecutive sequence of acid-induced dental hard tissue loss. Namely, the graduate loss of dental substance was observed in mandibular molars from sports drink, cola light, to cola drink, in a progressive order. The initial loss of enamel occurred on the occlusal surface, including the tip of the first and the second lingual cusps, which eventually resulted in the exposure of enamel. The distinct erosive step, generating the border between the affected and unaffected cervical enamel, was observable. As the erosive challenge persisted, the cervical progression of the erosive step was noticeable, as well as the initial loss of the enamel on the lingual third cusp was evident. Simultaneously, the first and the second lingual cusps exhibited more loss of dental substance in both the cervical and mesio-distal direction, towards the underlying dentine. The exposure of dentine at the top of the molar cusps resulted in a more rounded morphology of the teeth. After most of the enamel above the erosive step was eroded, the exposed underlying dentine appeared as a continuous layer with progressive erosion in the cervical, mesio-distal, and even pulpal direction. The continuous erosion of dentine in the pulpal direction resulted in

loss of the curved outline of the lingual tooth surface. The described pattern revealed a certain level of resemblance with erosive wear in human dentitions, where the typical cuppings on the occlusal surface of the molars are observed [200].

NOD mice develop salivary flow impairment approximately at 17 weeks of age, and histopathologic changes in the salivary glands of NOD mice have been observed as early as week 8 [172, 173]. All three upper molars from the NOD mice exhibited a characteristic erosive border lingually in the cola light and cola drink groups. This step was more accentuated compared to the findings in mice with normal salivary flow (Paper I). However, maxillary molars from the cola light groups were more affected compared to in the cola drink groups even though the latter displayed a greater overall loss of dental substance. The particular reason for this finding is not easily explained.

In the second study (Paper II), it was of great importance to use the animals at the age when they develop the salivary function impairment. However, the challenges were met during the study. Namely, 15 mice in total died during the experiment. NOD mice are prone to spontaneous diabetes development, resembling the human type 1 diabetes [201]. A study found that the incidence and progression of type 1 diabetes in NOD mice is correlated to the pH of drinking water as well as the gut microflora [202]. Predominantly the female NOD mice exposed to acidic water have a greater occurrence of insulinitis and hyperglycemia compared with those on neutral pH water [202]. Therefore, the lethal outcomes in the present study can be related to the fact that mice were exposed to acidic drinks. Even so, two deaths in the groups exposed only to distilled water with neutral pH were recorded. Thus, the possibility of spontaneously developed diabetes in NOD mice arose. Another marker indicating the probable development of diabetes in the mice was the weight gain. Although consuming approximately the same amounts of drinks as in study 1, the NOD mice exhibited a considerably lower mean weight gain compared to the CD-1 mice. Serum glucose levels in both healthy animals and

animals that exhibited abnormal behavior was recorded. We also found a decrease in the salivary flow rate related to age. Additionally, it was concluded that it was not influenced by the diet since it was similarly reduced in all NOD mice.

Since the study using NOD mice indicated the role of salivary flow rate in protecting against dental erosion, the intention with the third study (Paper III), was to investigate a possible relationship between salivary composition, AQP genes, and dental erosive wear severity in humans. Thereafter, the joined influence of AQP genes and selected environmental factors was explored. The results pointed at possible involvement of two aquaporins, AQP2 and AQP5, in the pathogenesis of dental erosive wear. Additionally, the amount of drinks consumed, and the frequency emerged as the variables involved in pathogenesis of the condition.

The use of AQPs targeted therapies has been suggested in order to modulate the inflammatory response of the host, as it is presumed that AQPs have a role in inflammatory processes, such as cell migration, the release of inflammatory cytokines and mediators, and edema [203]. The relationship of AQPs and dental caries showed a nominal association between caries experience and some *AQP* genes [204]. However, studies on their influence in the pathogenesis of dental erosive wear have been limited until now. Both genetic and environmental factors are involved in pathophysiology of diseases and conditions [151, 205]. Genetic analyses provide somewhat limited information about inherited conditions. A variation in genes can contribute to the increased or decreased susceptibility of enamel to mineral loss under acidic conditions. Our study comprised the environmental factors as covariates and the patients' erosion clinical data were broken down into distinct groups (phenotypes). Genetic components included nine SNVs located in the regions of the genes apparently related to the oral environment. The study population were adolescents of 16, 17, and 18 years of age. These age groups are suitable for such investigations for a certain number of reasons. Namely, dental erosive wear predominantly caused by acidic exposure is frequently found in younger

individuals [181]. Furthermore, the genetic role can be camouflaged by the domination of environmental factors with increasing age [206]. However, due to the narrow age span in the study population it is possible that the actual variation in the prevalence of dental erosive wear was not masked by environmental factors.

This study investigated the exposure of the dentition to the extrinsic sources of acid, and it would be convenient to examine the influence of intrinsic acids as well. Additionally, the patients in this study were classified according to the dental erosion degree, but not according to the acid exposure. However, a classification according to the acid exposure would give the opportunity to investigate the association of the chosen genetic variants and phenotypes of individuals with similar exposure but different degree of erosive wear.

Questionnaires are commonly used when collecting data of dietary habits. However, a minor limitation of this method is the patient's ability to recall when answering the questions. It is an advantage to have the answers collected prior to the informing the patient about the results of the clinical examination, in order to eliminate the information bias. Further, this study did not include data of salivary flow or buffering capacity of the individuals, as they were a homogenous group, i.e. young, healthy, without the reported use of medications that might alter the salivary flow.

Concluding Remarks

In Paper I of the present work, the obtained results supported the hypothesis that frequent exposure to acidic drinks can cause erosion in mice molars. The most importantly, an animal model of extrinsic dental erosions was established. In the second study (Paper II), we used mice with impaired salivary gland function as the animal model in order to investigate the influence of diminished salivary flow on the development of dental erosions. The results revealed positive association between erosive wear and reduced salivary flow. As it is desirable to have study designs as similar to those in human oral environment as possible, animal models are highly advantageous due to their salivary influence and soft tissue interactions. However, even though the NOD mice with impaired salivary flow represent a useful model in studies of the salivary contribution to dental erosive wear, the occurrence of diabetes in these mice may be a challenge for the researchers, and therefore, such experiments should be carefully designed.

The third study (Paper III) aimed to explore the connection between different dental erosive wear phenotypes in humans, aquaporins' genes, and certain factors from the environment. It was found that a combination of dietary preferences and variations in *AQPs*' genes are related to susceptibility to the dental erosive wear. Thereby, it may be concluded that the composition of the saliva influences the presence and severity of dental erosive wear. Some systemic diseases associated with impaired secretion of saliva, such as Sjögren's syndrome, diabetes mellitus type 1, and neurological conditions treated with donepezil, exhibit lower AQP5 levels [207]. Our findings are useful for both the dentists and physicians treating those patients since they may be classified as patients at particular risk for the development of dental erosive wear. It is important to bear in mind that early identification of any risk patient opens the doors for adequate and proper preventive treatment. Last but not the least, additional

knowledge about genetic-environmental interaction represent an important contributor to the general picture of dental erosive wear and serves as a precious platform for further research.

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Paper I



New animal model of extrinsic dental erosion-Erosive effect on the mouse molar teeth

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ABSTRACT

Objective: Consumption of acidic food and drinks is considered as important risk factor for development of dental erosion. There are several *in vitro* and *in situ* studies focusing on the risk indicators and preventive treatment, however, the need for a standardized animal model has been emphasised for many years. The aim was to establish an animal model of extrinsic dental erosion, which may serve as a standard for future studies to improve our understanding of the erosion.

Design: Two acidic drinks, sports drink and cola drink, were given to young mice for six weeks. Experimental and control (water) molars and incisors were dissected out and observed by scanning electron microscopy (SEM). Mandibular first molars were subsequently ground transversely and observed again by SEM. The tooth height and enamel thickness were measured on the SEM images.

Results: The lingual surface of the mandibular molars was most eroded after consumption of acidic drinks. The cola drink exhibited higher erosive effect on mandibular molars compared to sports drink. The lingual tooth height, compared to control, was about 34% and 18% lower in the cola drink and sports drink molars, respectively. Compared to the control molars, the lingual enamel was about 23% thinner in the sports drink molars and totally eroded on the certain lingual areas of the cola drink molars.

Conclusions: This new animal model of extrinsic dental erosion and the presented method with ground molars observed in SEM are suitable for further studies, which will gain deeper insights into the erosive disease.

1. Introduction

Several chemical and mechanical impacts contribute to the wear of the dentition throughout life. The manifestation of dental erosion, acid induced dental substance loss, has generally been accepted to be a multifactorial condition caused by various extrinsic and intrinsic acid sources (Lussi & Carvalho, 2014). There are indications that the prevalence of erosive tooth wear is increasing, especially in younger people, partly due to a change in nutritional habits and lifestyle (Jaeggi & Lussi, 2014; Mulic, Vidnes-Kopperud, Skaare, Tveit, & Young, 2012). A recent review and meta-analysis estimated the prevalence among

children and adolescents to be on average 30% (Salas, Nascimento, Huysmans, & Demarco, 2015). Extrinsic acids are mainly acidic drinks and food. Therefore, individuals consuming such products frequently are at risk for this type of dental hard tissue destruction. However, clinicians are still observing that dental erosion may occur or be absent regardless of these factors. The reason for that is still elusive, but it has been suggested that the individual's susceptibility to dental erosion is influenced by genetic variation (Chadwick et al., 2005; Sovik, Skudutyte-Rysstad, Tveit, Sandvik, & Mulic, 2015; Uhlen, Stenhagen et al., 2016), as well as by factors in the oral environment (Chadwick et al., 2005; Uhlen, Mulic, Holme, Tveit, & Stenhagen, 2016).

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Both the salivary flow rate and the composition of saliva are important factors that could have impact on its protective properties, and saliva has been considered as the most important biological factor in the prevention of dental erosion (Buzalaf, Hannas, & Kato, 2012; Hara & Zero, 2014). It has been suggested that the flow rate may be the best clinical indicator of the protective properties of saliva (Tenovou, 1997). As far as we know, there are only a few studies investigating the association of low salivary flow rate and the occurrence of dental erosions (Aldosari et al., 2018; Jarvinen, Rytomaa, & Heinonen, 1991; Jensdottir, Buchwald, Nauntofte, Hansen, & Bardow, 2013; Johansson, Norring, Unell, & Johansson, 2012; Mulic, Tveit, Songe, Sivertsen, & Skaare, 2012). Furthermore, different fluoride treatments in high concentrations are recommended as part of preventive treatment for individuals with risk for dental erosion. Conventional fluorides offer some, but limited protection against erosion (Magalhaes, Wiegand, Rios, Buzalaf, & Lussi, 2011). Therefore, the interest has grown into fluoride compounds containing polyvalent metal cations such as stannous fluoride (SnF₂) and titanium tetrafluoride (TiF₄). These agents have shown a protective, anti-erosion effect *in situ* (Schlueter, Klimek, & Ganss, 2009; Stenhagen, Hove, Holme, & Tveit, 2013). It has been concluded that tin-containing fluoride product might provide the best protection (Magalhaes et al., 2011).

Although there are a number of *in vitro* and *in situ* studies focusing on the risk indicators and preventive treatment of dental erosion, there are only few studies that have investigated the influence of certain risk indicators related to dental erosion in animal models (Aldosari et al., 2018; Sorvari & Kiviranta, 1988; Sorvari, Kiviranta, & Luoma, 1988; Sorvari, 1989; Sorvari, Peltari, & Meurman, 1996). Standardized *in vivo* models, which compared to *in vitro* and *in situ* studies without the saliva and soft tissue interactions, are suitable for further studies that may gain deeper insights into the salivary influence on development of dental erosive lesions. An advantage of an animal model, compared with human studies, is that the experimental procedures may be performed under more controlled conditions. Furthermore, human *in vivo* experiments are considered as unethical because of the irreversible loss of dental hard tissues. In the animal models used previously, the methods with limited possibility to study the details did not allow registration of small erosive lesions and their depths (Higo et al., 2009; Sorvari & Kiviranta, 1988).

The need for a standardized animal model for studying dental erosion has been emphasised for many years (Curzon & Hefferren, 2001). A new animal model where dental lesions of different severity can be created and analysed with sensitive methods is therefore warranted. The aim of the present study was to create an animal model of extrinsic dental erosion that will improve our understanding of erosive dental disease and serve as an appropriate model for future studies. For this purpose, experimental dental erosion was induced in mouse, and the erosive effect of products containing both citric (sports drink) and phosphoric (cola drink) acid on their dentition was studied in detail. We hypothesise that acidic drinks induce dental erosion in mouse teeth, and that comprehensive measurements of enamel loss and reduction in the tooth height may be recorded by SEM.

2. Materials and methods

2.1. Animal model

Ninety phenotypical, young female mice (CD-1 strain, 7 weeks old, 30 ± 5 g body wt) were selected for the study. Prior to experimental use, the animals were given standard laboratory fodder and water *ad libitum*, and they were maintained on a 12 h light: dark cycle, at 21 °C with a relative humidity of 65%. The animals were kept in accordance with Norwegian regulation and legislation (Norwegian Regulation on Animal Experimentation of 2015 based on EU directive on the Protection of Animals used for Scientific Purposes 2010/63/EU and Norwegian Animal Welfare Act of 2009). The experiment was approved

by Norwegian Food Safety Authority (FOTS ID 12710).

Before the experimental erosive procedures, the wire cages with solid bottom and bedding were prepared in order to reduce the wear of the dentition by attrition. The cages were carefully inspected before the mice were transferred into them. Any hard objects such as wooden sticks and plastic wheels were removed from the cages, and the animals were only supplied with paper boxes and paper ribbons as a part of environment enrichment. All cages were replaced two times per week, and the animals were monitored daily. Moreover, in order to reduce attrition of the teeth during the experiment, the standard laboratory fodder was softened prior to feeding. Fifty pieces of Teklad Global 18% Protein Rodent Diet (Envigo Teklad, Madison, WI, USA) were soaked with 165 ml of cold tap water, sealed in a plastic bag and left for softening overnight.

The mice were randomly distributed into three experimental groups, which were provided with distilled water (control), Red Bull sugar free sports drink (citric acid, pH = 3.39), and Coca Cola drink (phosphoric acid, pH = 2.27), respectively. Each group (n = 30 animals), was further divided into triplicate subgroups, i.e. ten animals per cage. Two 250 ml bottles with drinks were placed into each cage, and the bottles were replaced three times per week. Prior to the experiment, the changes in pH of both sports drink and cola drink were monitored over a period of three days, and the results showed no changes in pH. During the experiment, all animals were provided with softened laboratory fodder and drinks *ad libitum*, and the consumption of drinks in each cage was recorded. After the experimental period of six weeks, all animals were sacrificed by cervical dislocation, and their heads were fixed in 70% ethanol. All animals were weighted at the start and at the end of the experiment.

2.2. Scanning electron microscopy

The maxillary and mandibular molars and incisors were dissected out and fixed in 70% ethanol. The isolated teeth were thoroughly cleaned by dissection and by gentle brushing under running tap water. The specimens were air-dried overnight and mounted on brass cylinders with cyanoacrylate glue, sputter-coated with 30 nm platinum and observed in a Philips XL30 ESEM (Philips, FEI, Netherlands) operated at 12 kV.

The jaw segments containing all three molars were thereafter embedded in Epon and ground transversely. The grinding was performed under a stereo-microscope using grits 800 and 1200 3M waterproof silicone carbide paper (3 M, St. Paul, MN, USA) in a specially designed apparatus (Risnes, 1985). The ground surfaces were then polished by grinding the specimens against the backside of the 3M waterproof silicone carbide paper with 0.05 µm particle size alumina powder (Buehler Micropolish, Buehler, Lake Bluff, IL, USA) in water. After careful brushing under running tap water and removal of excess water, the teeth were etched for 45 s in 1% nitric acid, air-dried overnight, sputter-coated with 30 nm platinum and observed in scanning electron microscopy (SEM). For the transversely ground molars the whole procedure (grinding, polishing, etching, air-drying, sputter-coating, and observing in SEM) was repeated, creating two transversely ground planes for observation. The first plane (T1) was positioned on the mesial aspect of buccal cusp B2 and lingual cusp L2 where the tip of the cusps exhibited enamel-free areas (Lyngstadaas, Moinichen, & Risnes, 1998). The subsequent plane (T2) was ground further in distal direction ending on the distal aspect of buccal cusp B2 and lingual cusp L2 where the tip of the cusps was covered with enamel. The T1 and T2 planes were positioned in an area where the occurrence of dental erosions on the first molars were noted when the whole teeth were observed in the SEM.

2.3. Measurements and statistical analysis

SEM images of the transversely ground and etched plane T1 were

used for measurements of tooth height and enamel thickness in distilled water (control), sports drink, and cola drink mandibular first molars. Mean values and standard deviations were calculated using Microsoft Excel Worksheet. The step initiation at the eroded lingual enamel in sports drink and cola drink molars, was calculated by measuring the distance between the horizontal line at the level of the step and the corresponding line at the level of the enamel-cementum junction (illustrated in Fig. 5). Measurement data were tabulated and analyzed using the Statistical Package for Social Sciences 22.0 for Windows (SPSS Inc., Chicago, Illinois, USA). One-way analysis of variance (ANOVA) followed by the Tukey post-hoc test, and independent *t*-tests were used for the evaluation of data. *P*-values < 0.05 were considered statistically significant.

3. Results

3.1. Animal wellness and behavior

During the experimental period of six weeks, the animals exhibited normal behavior. Only one animal from the control group showed signs of behavioral disorder and illness four weeks after the start of the experiment, and was consequently excluded from the study. Daily consumption of drinks was about 50 ml per cage, and by the end of the experiment the mean weight gain of the animals was 3.71 g, 3.52 g, and 3.40 g in the control, sports drink, and cola drink group, respectively.

3.2. Tooth morphology and enamel distribution in control and experimental mice

The maxillary and mandibular jaw segments were observed from the occlusal, buccal, and lingual aspects. All three molars, in both the control and experimental mice were erupted and in occlusion (Figs. 1a, g, m & Fig. 2a, d, g). In the control mice, the molar enamel was unaffected with a smooth surface covering all sides of the teeth and forming a cuspal complex surrounding a common dentin surface at the tip of the cusps (Figs. 1a–f & 2a, b). The experimental mice exhibited various degrees of erosion mainly restricted to the lingual and occlusal part of the molars, being more evident in mandibular (Fig. 1g–r) compared to maxillary (Fig. 2d, e, g, h) molars. No morphological differences and effects of acid were observed between the control and the experimental incisors (Fig. 2c, f, i).

A specific erosion pattern was observed in both sports drink (Fig. 1g–l, Fig. 2 d, e) and cola drink (Fig. 1m–r, Fig. 2 g, h) molars, the latter being considerably more affected. There were no significant differences between molars on the right and left side of the mouth. In the mandibular sports drink molars, a distinct step on the lingual side was observed on all three molars, representing the border between the unaffected cervical and affected occlusal part of the teeth (Fig. 1g, i). The enamel on the buccal aspect of the teeth was unaffected (Fig. 1k, l). Both lingual and buccal cusps were reduced in height and showed a blunter appearance compared to control molars (Fig. 1i–l). The lingual enamel was eroded; however, it covered the dentin at all aspects of the cusps except for the enamel-free areas at the top (Fig. 1g–l). In the maxillary sports drink molars, a distinct erosion step on the lingual side was observed only on the first molar (Fig. 2d), particularly on the mesial half of the tooth including the mesial central cusp (Fig. 2e).

In the mandibular cola drink molars, severe erosion on the lingual side was observed on all three molars (Fig. 1m, o). The enamel was completely eroded on the lingual aspect of all lingual cusps with exposed and partially eroded dentin (Fig. 1m–p). The cervical part of the lingual enamel under the erosion step and the enamel on the buccal aspect of the teeth was not affected (Fig. 1o–r). Both lingual and buccal cusps were dramatically reduced in height, and the cusps exhibited a rounded appearance (Fig. 1m–r). On the occlusal aspect of the teeth, erosion had increased the size of the enamel-free areas, which were continuous with the lingual dentin (Fig. 1n). In the maxillary cola drink

molars, an erosion step on the lingual side was observed on the first and second molar (Fig. 2g). The enamel was slightly eroded on the lingual aspect of the second molar (Fig. 2g) and totally eroded on the lingual aspect of the first molar including the mesial central cusp (Fig. 2h).

3.3. Erosive effect on the dental enamel

Based on the SEM observations of the whole teeth (Figs. 1 & 2), the mandibular first molar was chosen as a model for further studies of dental erosion. Accordingly, two transversely grinded planes for observation (T1 and T2) were prepared, as described in Materials and methods. With the present tooth sections, detailed loss of enamel layers in experimental molars compared to control molars were recorded (Figs. 3–5, Table 1).

In Fig. 3, images from the transversely ground plane T1 of the mandibular first molars from the control, sports drink, and cola drink mice are presented. At this position, enamel-free areas at the tip of the cusps are normal morphological features (Fig. 3a, d, g). The unaffected enamel at the lingual aspect of control molars (Fig. 3a–c) and at the cervical part of the lingual aspect of experimental molars (Fig. 3d, e, g, h) exhibited a normal basic structure in both the control and experimental molars. The characteristic four layers: 1) an inner prism-free layer, 2) an inner enamel with prism decussation, 3) an outer enamel with parallel prisms, and 4) a superficial prism-free layer were observed. At about 274 μm , as measured from the enamel-cementum junction, erosive step was evident in the sports drink molars (Fig. 3d, e; Fig. 5 j; Table 1). Going from this step in an occlusal direction, the enamel was gradually lost, with complete loss of superficial enamel and partial loss of outer enamel (Fig. 3e, f). At this level, the enamel was about 23% thinner (52 μm vs 68 μm) in the sports drink molars compared to the control molars (Fig. 3b, c, e, f; Fig. 5 g, h; Table 1). The lingual tooth height was about 18% lower (637 μm vs 781 μm) in the sports drink molars compared to the control molars (Fig. 5a, b; Table 1). The enamel on the buccal aspect was unaffected, and the buccal tooth height was only slightly reduced (Fig. 5d, e; Table 1). Further distally, as shown in the transversely ground plane T2 (Fig. 4), the loss of enamel as observed at the level of the erosive step is even more steep, with initial loss of the superficial and part of the outer enamel (Fig. 4d, e). Immediately thereafter, a complete loss of outer enamel is evident (Fig. 4e, f). At this level, the cusps are normally covered with enamel (Fig. 4a, d).

The transversely ground planes of the first mandibular cola drink molars revealed a similar erosive pattern as observed in the sports drink molars, *i.e.* the lingual enamel being most affected (Figs. 3 & 4). However, the erosive effects in the cola drink molars were more severe, and more extended in the cervical direction (Fig. 3g; Fig. 5 k). The unaffected cervical enamel exhibited a normal basic four-layered structure (Fig. 3h). At T1, about 183 μm , as measured from the enamel-cementum junction, an erosive step was evident in the cola drink molars (Fig. 3g, h; Fig. 5 k; Table 1). Going from this step in an occlusal direction, the enamel was rapidly lost, with complete loss of superficial and outer enamel, and gradual loss of inner enamel (Fig. 3h, i). Further occlusally, the complete loss of enamel was evident (Fig. 3j, k). At this level, dentin erosion was also observed, as judged by the lingual outline of the dentin surface (Fig. 3g; Fig. 5). The tooth height was about 34% lower (512 μm vs 781 μm) in the cola drink molars compared to the control molars (Fig. 5a, c; Table 1). The enamel structure and thickness on the buccal aspect was unaffected (Figs. 3–5), however, the buccal tooth height was reduced with about 12% (513 μm vs 580 μm) (Fig. 5d, f; Table 1). Further distally, as shown in the transversely ground plane T2 (Fig. 4g–k), the loss of enamel exhibits the same patterns as observed at T1, but here the level of the erosive step is steeper, resembling the findings of the sports drink molars (Fig. 3h; Fig. 4 i).

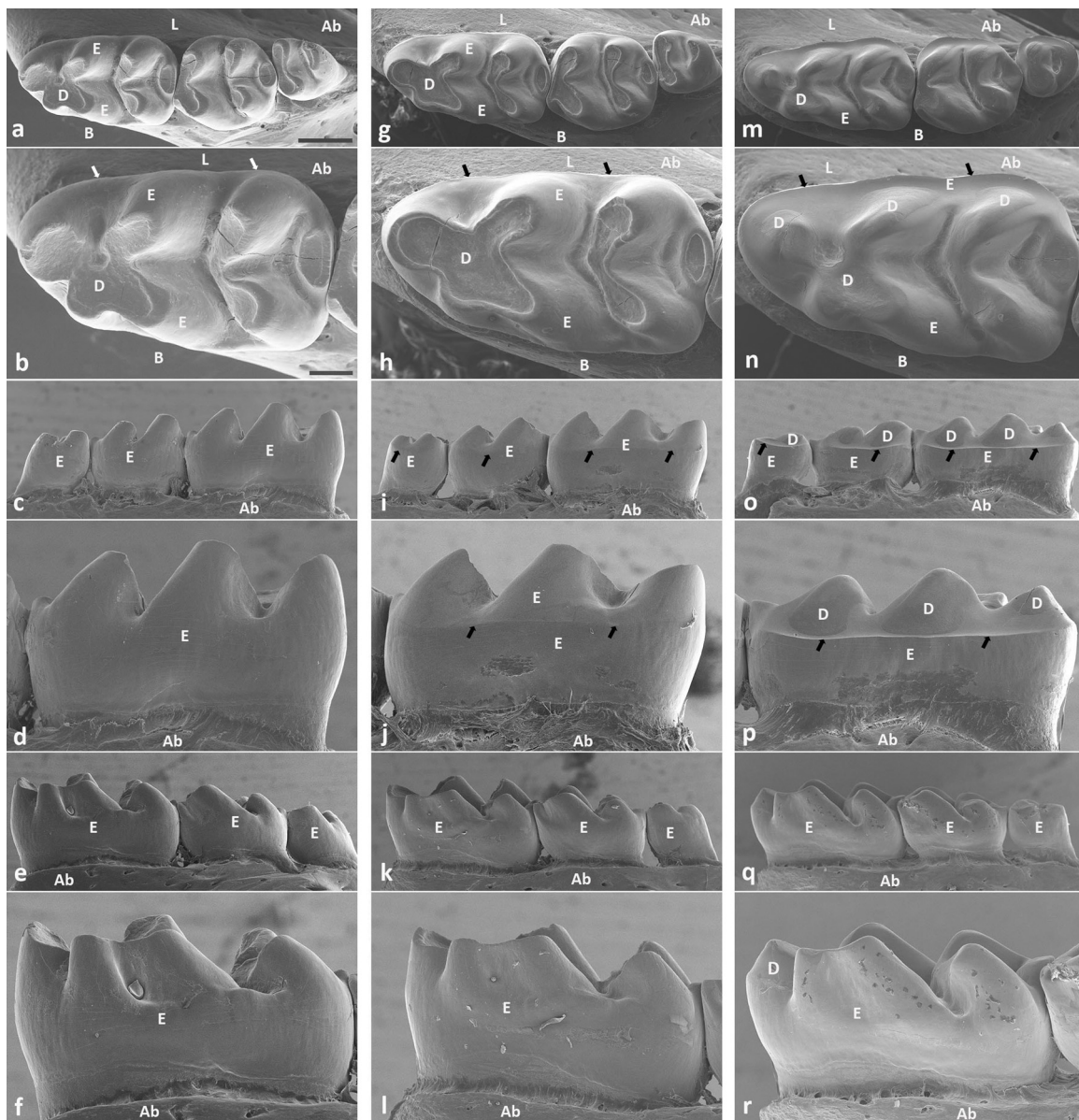


Fig. 1. Scanning electron microscopy images of *mandibular* molars from control (a–f), sports drink (g–l), and cola drink (m–r) mouse. Occlusal view (a, b, g, h, m, n), lingual view (c, d, i, j, o, p), and buccal view (e, f, k, l, q, r). (b, h, n) Higher magnification of occlusal view of mandibular first molar in panels a, g, m, respectively. (d, j, p) Higher magnification of lingual view of mandibular first molar in panels c, i, o, respectively. (f, l, r) Higher magnification of buccal view of mandibular first molar in panels e, k, q, respectively. The white arrows in panel b point to the unaffected and intact lingual surface of the first mandibular control molar. The black arrows in panels h, n, i, o, j, p point to the affected enamel on the lingual surfaces of the mandibular sports drink and cola drink molars. The step indicates the border between the unaffected cervical part and the affected occlusal part of the tooth. The bar represents 500 μm in panels a, g, m, c, i, o, e, k, q, and 200 μm in panels b, h, n, d, j, p, f, l, r. E = enamel, D = dentin, Ab = alveolar bone, B = buccal side, L = lingual side.

4. Discussion

Many dietary drinks include different types of acids, such as citric acid, phosphoric acid, carbonic acid, ascorbic acid, and malic acid, which has an impact on the erosive effect (Barbour & Lussi, 2014; Shellis, Featherstone, & Lussi, 2014; Zero & Lussi, 2005). Lifestyle, drinking habits, and the method of consuming drinks differ between individuals and are accordingly important for how long acids are retained in the mouth before swallowing. It has also been shown that the drinking technique, as well as amount are of consequence for the risk of developing erosive lesion (A. K. Johansson, Lingstrom, Imfeld, & Birkhed, 2004; Sovik et al., 2015). In the present animal study, two popular drinks among both adults and adolescents were chosen, *i.e.* sports drink (citric acid, pH = 3.39) and cola drink (phosphoric acid, pH = 2.27). The mice were served these drinks as the only drink for six

weeks and, thereafter, the erosive effect on the whole dentition was studied. The results demonstrated a specific erosion pattern, similar to lesions seen in patients, in both the sports drink and cola drink molars, the latter being considerably more affected. The findings showed, as well as in previous animal studies (Gortner et al., 1945; Sorvari et al., 1988; Spencer & Ellis, 1950), small individual variation in the erosive effects between mice within the same experimental group. This variation may be due to different drinking habits and the amount of drink consumption, which were not possible to control during the experiment. However, by observing the mice daily during the experimental period of six weeks, no special differences could be observed in drinking frequency. Furthermore, these small differences within the same group of mice may be due to other individual factors, *e.g.* salivary flow and saliva buffering function.

In accordance with previous studies in rats (Sorvari & Kiviranta,

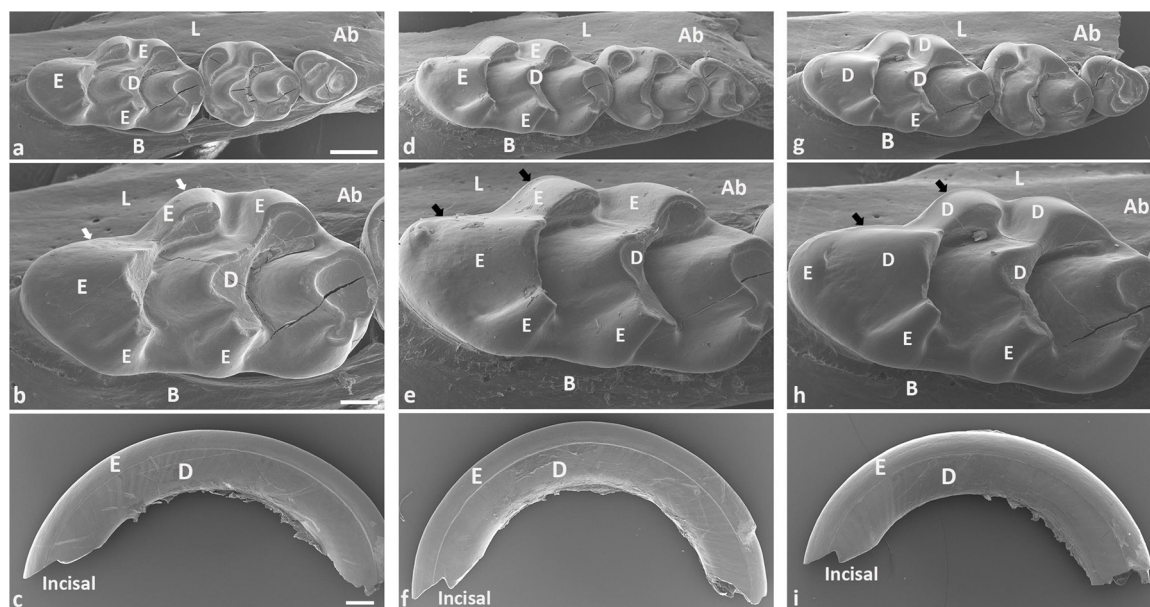


Fig. 2. Scanning electron microscopy images of *maxillary* molars and incisor from control (a-c), sports drink (d-f), and cola drink (g-i) mouse. (a, d, g) Occlusal view of all three maxillary molars from control, sports drink, and cola drink mice, respectively. (b, e, h) Higher magnification of occlusal view of mandibular first molar in panels a, d, g, respectively. The white arrows in panel b point to the unaffected and intact lingual surface of the first maxillary control molar. The black arrows in panels e and h point to the affected enamel on the lingual surface of the first maxillary sports drink and cola drink molars. The step indicates the border between the unaffected cervical part and the affected occlusal part of the tooth. (c, f, i) Maxillary incisors from control, sports drink, and cola drink mouse, respectively. The bar represents 500 μ m in panels a, d, g, 200 μ m in panels b, e, h, and 500 μ m in panels c, f, i. E = enamel, D = dentin, Ab = alveolar bone, B = buccal side, L = lingual side.

1988; Sorvari et al., 1988, 1996) the lingual surface of mouse mandibular molars exhibited the strongest erosive effect after consumption of acidic drinks (Figs. 1 & 2). In addition, no significant differences between the first and the second and third mandibular molars (Fig. 1) were demonstrated. In the maxillary molars, which in general were less affected, the first molars were more affected compared to the second and third molars (Fig. 2). This is also reported in several clinical studies (Arnadottir et al., 2010; Ganss, Klimek, & Giese, 2001).

Previously, first rat mandibular molars have been preferred for studying erosion in animal models, mainly because of their size (Aldosari et al., 2018; Sorvari & Kiviranta, 1988; Sorvari et al., 1988, 1996). So far, few methods, with limited possibility to study the details, have been used in order to study the effects of acidic drinks on dental erosion. Already in 1988, the erosive effect on the rat molar teeth of a sport drink mixture with and without addition of fluoride and magnesium was studied (Sorvari et al., 1988). In that study, the lingual surfaces of the first mandibular molars were investigated and drawn under a stereomicroscope equipped with a drawing tube. Thereafter, the teeth were photographed with a camera, and then the total surface areas, intact areas, and eroded enamel were scored by a grading system (Sorvari et al., 1988). In a subsequent study by Sorvari and Kiviranta the method for erosion recoding was semi-quantitative, based on the subjective evaluation resulting in numerical data of the intact and eroded surface areas (Sorvari & Kiviranta, 1988). With this method, compared to the grading system used earlier, it was possible to compare total surface areas, which reflected tooth wear, especially occlusal wear. The measured size of the eroded area and the estimated area of occlusal wear together gave a more complete idea of the effects of an erosive agent than the methods used before. Ten years later, surface ultrastructure, particularly of dentin, was studied with SEM in rat molars after experimentally induced erosion and attrition (Sorvari et al., 1996). A recent study on rats was performed with the intention to investigate the susceptibility to dental erosion in partially desalivated animals. The loss of dental hard tissue was measured by use of micro CT (Aldosari et al., 2018). However, the present study is the first of its kind to investigate and standardize the effects of induced experimental

erosion in mice, which allows comprehensive measurements of enamel thickness and tooth size. In addition, the erosive effect on the enamel layers in different experimental groups was measured. In excessive tooth tissue loss where erosion, attrition, and abrasion occur simultaneously the original causes may be difficult to distinguish (Eccles, 1982; Shellis & Addy, 2014) and especially erosion may be overlooked in favor of attrition (Lewis & Smith, 1973). It is known that commercial pellets for laboratory animals are abrasive and increase tooth wear when compared with powdered diet, and the faster rate of attrition is not only due to abrasives in the hard pellets but also due to increased time of chewing (Teaford & Oyen, 1989). Additionally, a previous study has shown that different diets given to the rats for six weeks caused morphological surface changes, which were observed by SEM (Sorvari et al., 1996). Rough pellet food caused severe attrition on the lingual surface of the first mandibular molar; the surface was worn with the presence of scratches and small pits when compared to the teeth of rats given soft powdered diet. Studies have reported that erosion increases the susceptibility of the tooth surface to attrition and abrasion (Lewis & Smith, 1973; Shellis & Addy, 2014; Smith, 1975), and that erosive sport drink together with rough pellet food caused greatest tooth surface loss (A. Johansson, Haraldson, Omar, Kiliaridis, & Carlsson, 1993; Lewis & Smith, 1973; Sorvari & Kiviranta, 1988). In animal studies, abrasion can be excluded and, thus, the mechanical wear is mainly due to attrition. In our study, in order to reduce attrition of the teeth during the experiment, standard laboratory fodder was softened with water prior to feeding. However, a minor reduction of cuspal height due to occlusal wear was observed even in mice that were exposed only to water (Fig. 1a–f), as judged by cuspal complex surrounding a common dentin surface at the tip of the cusps, when compared to just erupted and non-worn mouse molars (Lyngstadaas et al., 1998). It is likely that some of this occlusal wear occurred before the start of the experiment at the age of seven weeks, since all molars in mice reach the occlusion at an age of 35 days (Lyngstadaas et al., 1998). Furthermore, some of the wear may have occurred during the time of the experiment, despite of exposure only to softened food. Importantly, all animals in this study, in both control and experimental groups, were given the same fodder, both

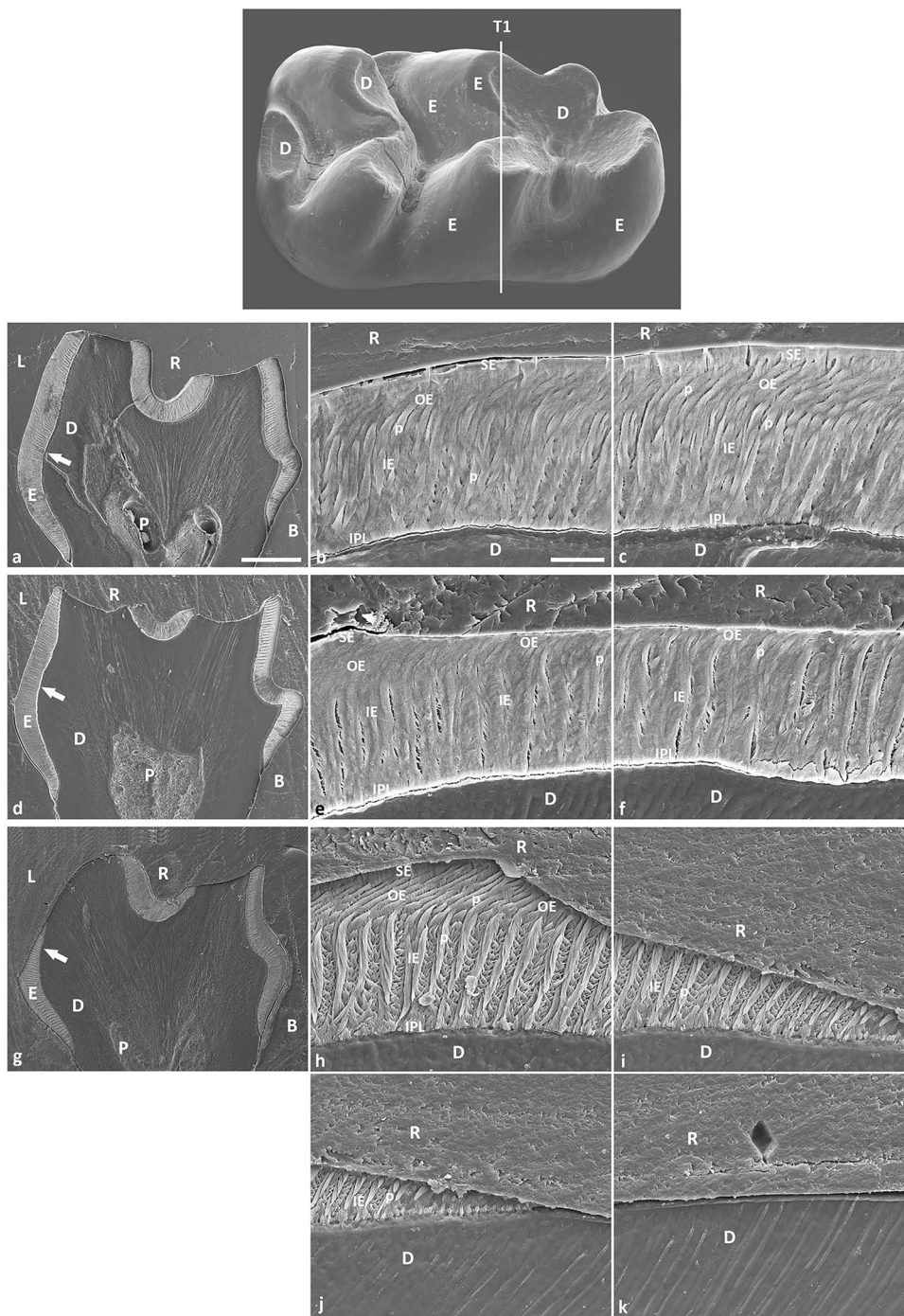


Fig. 3. Scanning electron microscopy images of transversely ground planes of mandibular first molar from control (a–c), sports drink (d–f), and cola drink (g–k) mouse.

The top picture shows a representation of a mandibular first molar, indicating the position of the transversely ground plane T1. The white arrows in panel a, d, g point to the lingual enamel, which is unaffected in panel a and affected in panel d and g. (b, c) (e, f) (h–k) Higher magnification of enamel at the position of white arrows in panel a, d, g, respectively. In the control molar the lingual enamel exhibits full thickness and normal basic enamel structure with four layers (b, c). In the sports drink molar, the lingual enamel exhibits a distinct step indicating the start position of enamel erosion (d, e). In the cola drink molar, the lingual enamel shows a distinct step with dramatic erosion of enamel in an occlusal direction (g–i), and thereafter a complete loss of enamel (j, k). The bar represents 200 μm in panels a, d, g, and 20 μm in panels b, c, e, f, h–k. E = enamel, D = dentin, P = pulp, R = resin, B = buccal side, L = lingual side, IPL = inner prism-free layer, IE = inner enamel, OE = outer enamel, SE = superficial enamel, p = prism.

before and during the experiment. Although, we cannot exclude a small influence of attrition on the occlusal aspects of the sports drink and cola drink molars, the findings observed in the enamel on the lingual part of the tooth, are predominantly due to erosion.

It is also worth mentioning, that in our model, rapid destruction of the tooth surface was also due to the exaggerative conditions, where the mice were exposed to acidic drinks continuously for six weeks. Therefore, extrapolating findings from the present study to humans must be taken carefully. Rodent and human enamel exhibits the same basic structural elements, prism and interprism, however, the spatial arrangement of the prisms, *i.e.* the prism pattern, is considerably different (Warshawsky, Josephsen, Thylstrup, & Fejerskov, 1981). Other differences include the speed at which enamel formation occurs (Risnes, 1979) and the incorporation of iron in the superficial enamel layer of

rodent incisors (Warshawsky & Smith, 1974). However, compared to several previously published *in vitro* and *in situ* studies, animal models are highly advantageous due to the salivary influence and soft tissue interactions, resembling the human oral environment.

The present results showed that the mandibular molars exhibited the strongest erosive effect after consumption of acidic drinks (Fig. 1). The maxillary molars were only slightly affected, especially the first molar, and the incisors were unaffected (Fig. 2). This is also reported in several clinical studies (Mulic, Tveit, & Skaare, 2013; Sovik et al., 2015). Mandibular molars, compared to maxillary molars, were probably more affected due to anatomical relations where the acid is present for a longer time in the sublingual compared to palatal area of the oral cavity. However, it is remarkable that when subjected to the cola drink, mandibular molars, which exhibited severe erosive defects lingually

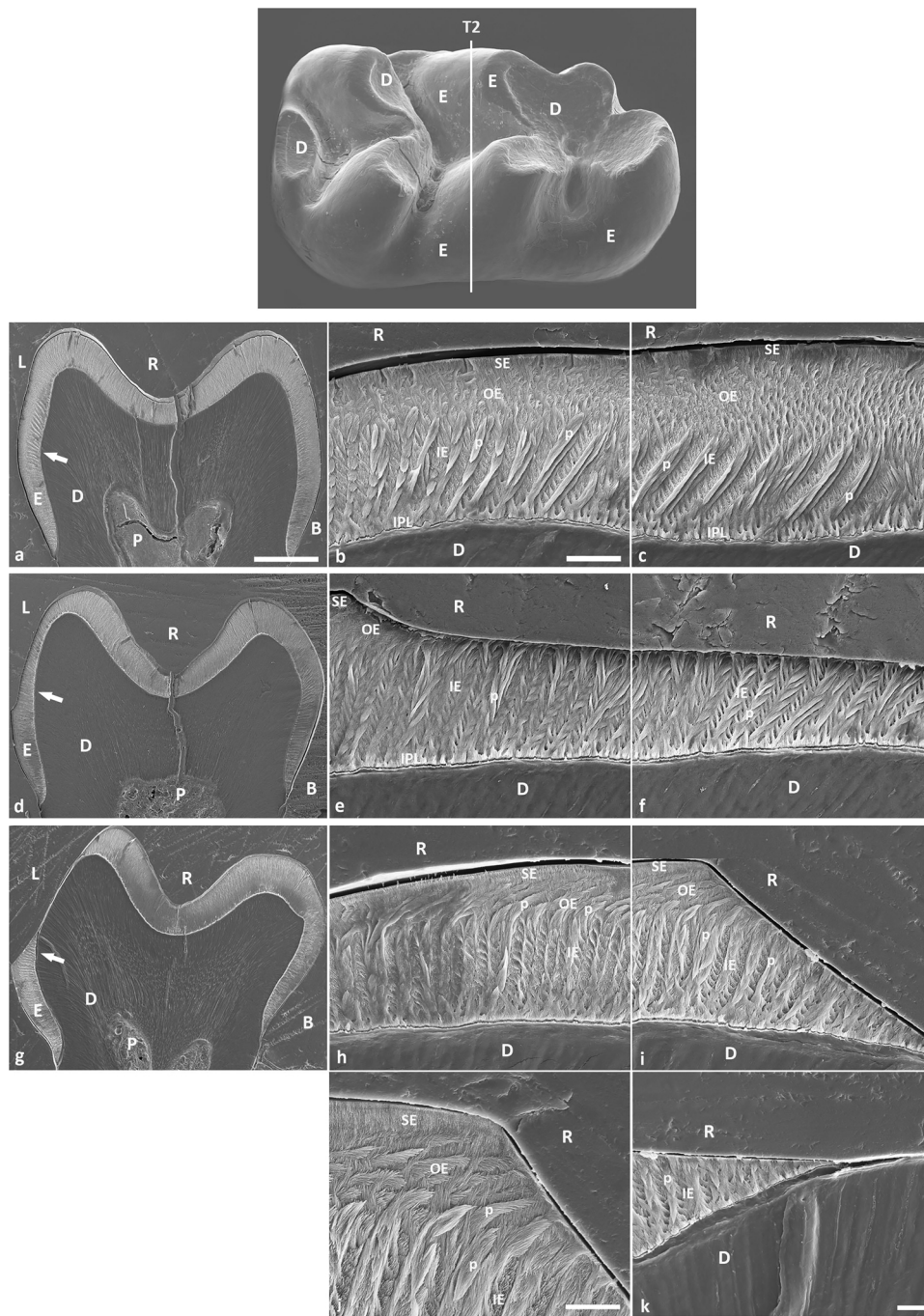


Fig. 4. Scanning electron microscopy images of transversely ground planes of mandibular first molar from control (a–c), sports drink (d–f), and cola drink (g–k) mouse.

The top picture shows a representation of a mandibular first molar, indicating the position of the transversely ground plane T2, which is situated further distally compared to T1. The white arrows in panel a, d, g point to the lingual enamel, which is unaffected in panel a and affected in panel d and g. (b, c) (e, f) (h–k) Higher magnification of enamel at the position of white arrows in panel a, d, g, respectively. In the control molar the lingual enamel exhibits full thickness and normal basic enamel structure with four layers (b, c). In the sports drink molar, the lingual enamel exhibits a distinct step indicating the start position of enamel erosion (d, e). The superficial and outer enamel is completely eroded (f). In the cola drink molar, the lingual enamel shows a distinct step with dramatic erosion of enamel in an occlusal direction (h, i), and thereafter a complete loss of enamel (j, k). The bar represents 200 μm in panels a, d, g, 20 μm in panels b, c, e, f, h, i, and 10 μm in panels j and k. E = enamel, SE = superficial enamel, p = prism.

and occlusally, showed no effects on the buccal surface (Figs. 1, 3 & 4). A likely explanation for this is that the acid did not remain in the mucobuccal fold long enough to induce erosion on the buccal aspect of the molars, in the same way as the acid in the sublingual area induced erosion on the lingual aspect of the same teeth. As expected, the mouse incisors were not affected, probably due to two main reasons. Firstly, it is not expected that the erupted part of the continuously growing mouse incisor, which exhibits enamel only on the labial aspect of the tooth (Sidaly, Risnes, Khan, Stiris, & Sehic, 2015), is in contact with a substantial amount of acid for a long enough time. Secondly, the enamel of mouse incisors, especially maxillary incisors, exhibits yellow-brown iron pigmentation, which is more resistant to acid (Moinichen, Lyngstadaas, & Risnes, 1996).

The results also demonstrated a very distinct step in the enamel on the lingual aspect representing the border between the unaffected

cervical part and the affected occlusal part of the molars (Figs. 3–5). It was striking that this cervical part of the enamel was not affected in the cola drink molars where the adjacent enamel was eroded in total (Fig. 4g–k). Previous observations have shown the same erosive pattern in rat molars (Sorvari et al., 1988). Gingiva covers a part of this cervical enamel, which may serve as a protection, however, a steep erosive gradient as observed in the cola drink molars (Fig. 3 & 4) is still elusive. One would expect the enamel layers to be gradually eroded along the whole surface that was exposed to acid.

The loss of enamel layers on the lingual aspect of the tooth is best observed in transversely ground planes through the tooth. Achieving ideal transversal ground plane of mouse molars through the correct cusps is technically difficult due to their small size, and consequently, the position of transversal sections may have varied slightly. However, it was considered unlikely that this slight variation would mask the

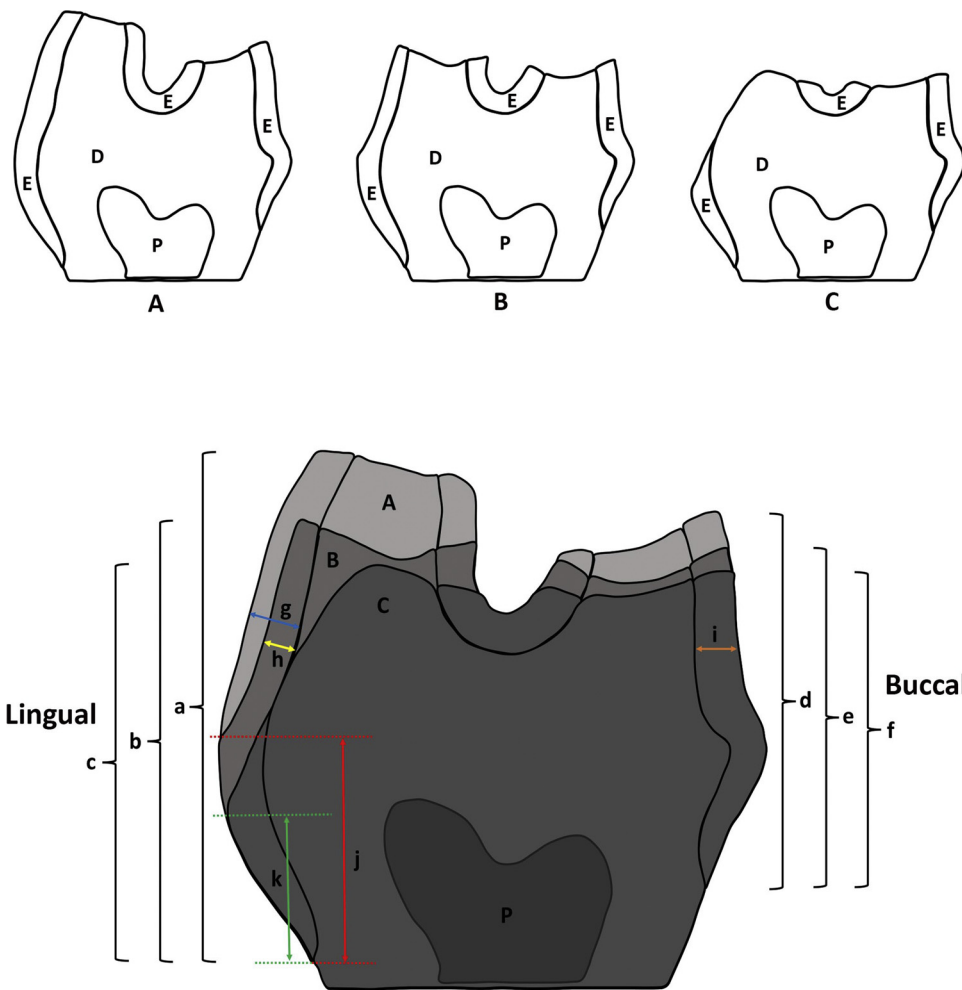


Fig. 5. Schematic representation of a transversely ground planes of mandibular first molar from control (A), sports drink (B), and cola drink (C) mouse.

The top picture shows a separate schematic presentation of transversely ground planes (T1 in Fig. 3) of mandibular first molar from each group (A–C). At the bottom is a collective presentation of ground molar planes from all three groups (A–C). Lingual tooth height: Control (a), sports drink (b), and cola drink (c). Buccal tooth height: Control (d), sports drink (e), and cola drink (f). The thickness of lingual enamel: control (g) and sports drink (h). The thickness of buccal enamel in all groups: (i). The distance of step initiation as measured from enamel-cementum junction in sports drink (j) and cola drink (k) molars. Dimensions are presented in Table 1.

Table 1
Dimensions of tooth height and enamel thickness.

	Control	Sports drink	Cola drink
Lingual tooth height	781 ± 11 (a)	637 ± 9 (b)*	512 ± 12 (c)*
Buccal tooth height	580 ± 7 (d)	539 ± 6 (e)	513 ± 9 (f)*
Lingual enamel thickness	68 ± 3 (g)	52 ± 2 (h)*	–
Buccal enamel thickness	75 ± 2 (i)	74 ± 2 (i)	75 ± 3 (i)
Erosive step	–	274 ± 8 (j)	183 ± 6 (k)

Measured dimensions (mean ± SD, μm) of mandibular first molar tooth height and enamel thickness are presented. Letters in parentheses refer to the letters in Fig. 5. The values represent measurements taken from SEM images of transversely ground plane T1 (Fig. 3).

(-) Not applicable; (*) Significant difference, $p < 0.05$.

significant differences in enamel thickness between the groups. In order to minimize contributions from random errors, samples from 30 mice including biological and technical triplicates were used for each group. It is also likely that there is some variation in the tooth size between the mice. However, previous studies from our lab have shown that these are minor in mice where the body weight is not significantly different (Lyngstadaas et al., 1998; Sehic, Peterkova, Lesot, & Risnes, 2009; Sehic, Risnes, Khuu, Khan, & Osmundsen, 2011; Sehic, Nirvani, & Risnes, 2013). Therefore, using CD-1 strain where the morphology and size of the mouse molars have previously been thoroughly described (Lyngstadaas et al., 1998), and including water as a control, we assume that this may not have affected the accuracy of the measurements significantly.

In the present study, two popular beverages were chosen, one sugar

free (sports drink) and the other containing sugar (cola drink). It may be speculated that sugar may have had some influence on the results; however, we did not observe by SEM any caries in the molars of the cola drink mice. Based on these results, it was evident that cola drink exhibited higher erosive potential compared to sports drink. The rate of dissolution of dental minerals, which is crucial to the progression of erosion, is influenced by solubility and also by other factors (Shellis et al., 2014). It is influenced strongly by type of acid, physical factors (temperature, flow rate) and chemical factors (degree of saturation, presence of inhibitors, buffering, pH, fluoride) (Shellis et al., 2014). Citric acid has a greater erosive potential than phosphoric acid, presumably related to its ability to form chelating complexes and due to its high buffering capacity (Zero & Lussi, 2005). Chelation is a process widely discussed in relation to erosive tooth wear, and is referred to as the ability of certain ions, particularly citrate, to bind calcium and form calcium complexes, and thereby favoring demineralization by increasing the degree of undersaturation (Zero & Lussi, 2005). Therefore, one would expect that the erosive effect of sports drink was higher compared to cola drink, however, the present results showed the opposite trend. It is difficult to isolate the chelating effect from the other parameters; thus, the connection is still uncertain. A recent study demonstrated that under flowing conditions, chelating effects of citric acid seems to be of negligible relevance with respect to enamel erosion (Azadi-Schossig, Becker, & Attin, 2016). The chemistry of dental erosion is complicated and involves several factors; however, it seems that the low pH of cola drink was an important factor for developing dental erosion in mouse molars.

5. Conclusions

Human experiments on dental erosion are unethical because of the irreversible loss of dental hard tissues when exposed to acids. Therefore, studies using animal models are of high importance since they represent clinical situations. Based on the present results it can be concluded that cola drink (phosphoric acid, pH = 2.27) exhibited higher erosive effect on mouse mandibular molars compared to sports drink (citric acid, pH = 3.39). This new animal model of extrinsic dental erosion where lesions of different severity can be created is suitable for further studies that will improve our understanding of the disease. The present method with transversely ground molars observed in SEM allows a registration of erosive lesions and lesion depths in small teeth like mouse molars. Both studies on the salivary influence (e.g. by using knockout mice) and the studies on the effect of fluoride on development of erosive lesions are warranted.

Conflict of interest

The authors declare no conflicts of interest, financial or otherwise.

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Paper II

II

Dental erosion in mice with impaired salivary gland function

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ABSTRACT

Objective: Salivary flow rate exerts an essential impact on the development and progression of dental erosion. In this work, the experimental dental erosion in non-obese diabetic (NOD) mice with reduced salivary flow rate was induced, and the erosive effect of acidic drinks on their dentition was studied.

Material and methods: Three acidic drinks (sports drink, cola light drink and sugar containing cola drink) were given to adult NOD mice (groups: $N=11$) as the only drink for 6 weeks. Two control groups were included; wild type and NOD control (groups: $N=9$). Experimental and control (water) teeth were dissected out and observed by scanning electron microscopy (SEM). Mandibular first molars were subsequently embedded in Epon, ground transversely, observed again by SEM, and the enamel thickness and tooth height were measured.

Results: Mandibular molars were considerably more eroded than maxillary molars. The erosive process started at the top of the cusps and subsequently extended in the cervical, mesio-distal, and pulpal direction. Erosive lesions were evident in increased succession from sports drink, cola light to cola drink exposed mandibular molars, with the lingual tooth height being approximately 23%, 26%, and 37% lower, respectively, compared to the control. The lingual enamel was approximately 48% thinner in sports drink molars and 62% thinner in cola light molars. In cola drink molars, the lingual enamel was totally eroded, and significant erosion of dentine was evident.

Conclusion: Reduced salivary flow, together with a high consumption of acidic drinks, results in severe erosion of NOD mice molars.

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Introduction

Dental erosion, or acid-induced dental hard tissue destruction, is a multifactorial condition caused by various extrinsic and intrinsic acid sources [1]. The prevalence of erosive tooth wear is increasing, mainly due to changes in lifestyle and drinking habits [2,3]. Individuals that frequently consume acidic drinks and food exhibit a higher risk for this type of dental substance loss, and the consumption of drinks and food with low pH has increased significantly over the past decades [4–6].

Understanding of dental erosive disease has improved considerably; however, knowledge of the role of factors in the oral environment, such as saliva and dental enamel, remains elusive. Human *in vivo* studies on dental erosion are unethical because of the irreversible acid-induced loss of dental hard tissues. Therefore, studies using animal models, in which the experimental procedures can be performed under controlled conditions, are important. However, only a few studies have focussed on the risk indicators and preventive treatment related to dental erosion in animal models [7–11], and the criteria for the registration of dental erosive

lesions were mainly semi-quantitative, and did not allow precise recording of small erosive lesions and their depth [9,12]. Recently, an animal model in which experimental dental erosion was induced in mouse with normal salivary flow was developed, and the erosive effect of products containing both citric and phosphoric acid was studied in detail [13]. The results showed that cola drink (phosphoric acid) exhibited higher erosive effects on mouse mandibular molars compared to sports drink (citric acid), and the presented method, with transversely ground molars observed under a scanning electron microscope (SEM), enabled comprehensive registration of erosive lesions and their depths in small teeth such as mouse molars [13].

Saliva, enamel phenotype, and dietary habits play essential roles in the formation and progression of dental erosion [14]. Saliva is considered the most significant biological factor in the prevention of and in modifying the development of dental erosive wear, and both the saliva composition and salivary flow rate are fundamental factors that may have an effect on its protective properties [15,16]. Saliva directly dilutes, clears, neutralizes, and buffers erosive agents; forms a protective membrane covering the enamel surface; reduces

the demineralization rate; and enhances the remineralization of eroded enamel and dentine by providing fluoride, phosphate, and calcium [15]. The salivary flow rate has been considered the best clinical indicator of the protective properties of saliva [17]; however, only a few studies have investigated the association between low salivary flow rate and the occurrence of dental erosive lesions [11,18–21].

Non-obese diabetic (NOD) mice are a well-known animal model for studying Sjögren's syndrome, an autoimmune disease characterized by ocular and oral dryness [22,23]. In NOD mice, salivary glands are most frequently affected with lymphocytic infiltrates, which lead to glandular hypofunction [24]. The very first signs of salivary gland infiltration have been observed at 8 weeks of age [25–27]. A study investigating salivary gland function in NOD mice reported significantly reduced salivary flow rate and a moderate presence of inflammatory cells in the salivary gland tissue between week 17 and 24 [28]. Another study reported progressive loss of salivary function between week 16 and 20 [29]. In this work, experimental dental erosion was induced in NOD mice and the erosive effect of products containing both citric (sports drink) and phosphoric (cola/cola light drink) acid on their dentition was studied in detail. The hypothesis was that acidic drinks induce dental erosion in NOD mice and that decreased salivary flow rate plays a role in this development of erosive lesions.

Material and methods

Animal model

Forty-two phenotypical 9 weeks old NOD/MrkTac female mice were purchased from Taconic Biosciences (Ejby, Denmark), and nine young wild-type female mice (CD-1 strain, 9 weeks old) were selected as an additional control. The animals were kept in accordance with Norwegian regulations and legislation (Norwegian Regulation on Animal Experimentation of 2015 based on EU directive on the Protection of Animals used for Scientific Purposes 2010/63/EU and the Norwegian Animal Welfare Act of 2009). They were maintained on a 12-h light:dark cycle at 21 °C with 65% relative humidity. The study was approved by the Norwegian Food Safety Authority (FOTS ID 16721).

Prior to experimental use, the NOD mice were kept in the facility for approximately 7 weeks for them to reach the age of 16 weeks, the time point at which they develop dysfunction of the salivary glands resulting in significantly reduced salivary flow rate [28,29]. Pilocarpine-stimulated salivary flow was measured in both wild type and NOD mice at 9, 17, and 22 weeks of age. Before the experimental erosive procedures, the animals were given water *ad libitum*. However, in order to exclude attrition of the teeth, both before and during the experiment, the standard laboratory fodder was softened prior to feeding. Fifty pieces of Teklad Global 18% Protein Rodent Diet (Envigo Teklad, Madison, WI, USA) were soaked with 165 mL cold tap water, sealed in a plastic bag, and left to soften overnight. Furthermore, wire cages with solid bottom and bedding were prepared to reduce wear of the dentition by attrition, as described previously [13]. The cages

were carefully inspected before the mice were transferred into them. Any hard objects such as wooden sticks and plastic wheels were removed from the cages, and the animals were only supplied with paper boxes and paper ribbons as part of the environment enrichment. All cages were replaced two times per week, and the animals were monitored daily.

At the age of 16 weeks, the NOD mice were randomly distributed into four groups, which were provided with distilled water (control) ($n=9$), Red Bull sugar-free sports drink (citric acid, pH 3.39) ($n=11$), Coca Cola light drink (phosphoric acid, pH 3.10) ($n=11$), and Coca Cola drink (phosphoric acid, pH 2.27) ($n=11$), respectively (Figure 1). The wild-type mice ($n=9$), serving as an additional control group, were provided with distilled water (Figure 1). Each group was further divided into triplicate subgroups (cages), and two 150-mL bottles with drinks were placed into each cage. The animals were provided with drinks *ad libitum*, the bottles were replaced three times per week, and the consumption of drinks in each cage was recorded. Prior to the experiment, the changes in pH of both the sports drink and cola drink were monitored over 3 days, and the results showed no changes in pH. After the 6-week experimental period, the animals were sacrificed at the age of 22 weeks by cervical dislocation, and their heads were fixed in 70% ethanol. All animals were weighed at the start and end of the experiment. Figure 1 presents the flow chart of the experiment.

Scanning electron microscopy

The maxillary and mandibular molars and incisors from both wild-type control, NOD control and experimental mice were dissected out and fixed in 70% ethanol. The isolated teeth were thoroughly cleaned by dissection and by gentle brushing under running tap water. The specimens were air-dried overnight and mounted on brass cylinders with cyanoacrylate glue, sputter-coated with 30 nm platinum and observed under a GeminiSEM 300 SEM (Zeiss, Oberkochen, Germany), operated at 5 kV.

Thereafter, the jaw segments with all three molars were embedded in Epon and ground transversely. Under a stereomicroscope, grinding was performed using grit 800 and 1200 waterproof silicon carbide paper (3 M, St. Paul, MN, USA) in a specially designed apparatus [30]. When the grinding reached the planned position, the surfaces were polished by grinding specimens against the backside of the silicon carbide paper with 0.05- μm particle size alumina powder (Buehler MicroPolish, Buehler, Lake Bluff, IL, USA) in water. After careful cleaning by brushing under running tap water and removal of excess water, the teeth were etched for 45 s in 1% nitric acid, air-dried overnight, sputter-coated with 30 nm platinum, and again observed under SEM. The whole procedure (grinding, polishing, etching, air-drying, sputter-coating, SEM observation) was repeated, creating three transversely ground planes for observation. The first plane (T1) was positioned on the mesial aspect of buccal cusp B2 and lingual cusp L2, where the tips of the cusps exhibit enamel-free areas [31]. The subsequent plane (T2) was ground further in the distal direction, ending on the distal aspect of buccal cusp B2 and lingual cusp L2, and the final third plane

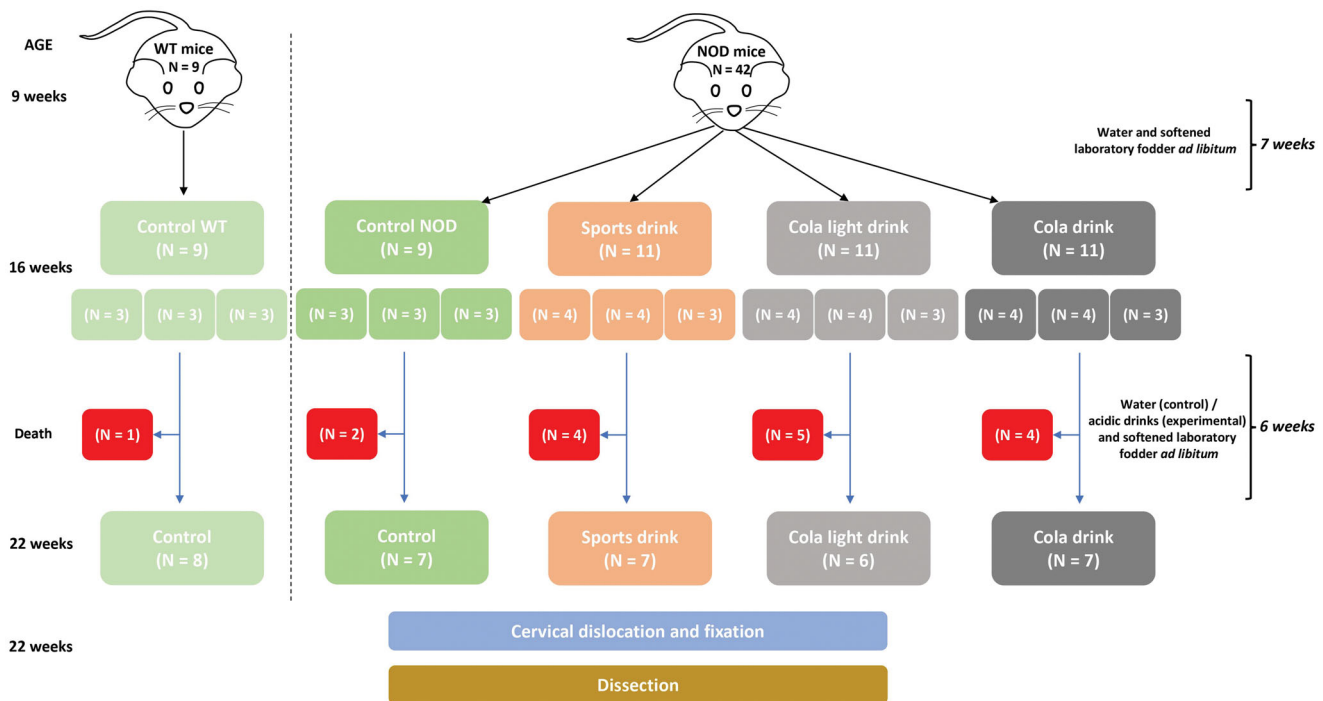


Figure 1. Flow chart of animals enrolled in the study. Forty-two NOD mice and nine wild-type mice, 9 weeks old, were kept for 7 weeks and provided with water and *softened* laboratory fodder *ad libitum*. At the age of 16 weeks, NOD mice were randomly distributed into four groups, i.e. control and three experimental groups. The wild-type group served as an additional control. After the 6-week experimental period, the surviving animals were terminated at the age of 22 weeks by cervical dislocation, and their heads were fixed in 70% ethanol.

(T3) was positioned reaching the mesial aspect of the buccal cusp B3 and lingual cusp L3. Figure 4 shows the position of transversely ground planes T1–T3. Since there were no morphological differences and trace of acid effect on the incisors, as judged by SEM, the incisors were not subject for grinding procedures.

Statistical analysis

SEM images of the transversely ground and etched plane T1 were used for measuring enamel thickness and tooth height in wild-type control, NOD control and experimental mandibular first molars, corresponding to our previous study [13]. Mean values and standard deviations were calculated using Microsoft Excel Worksheet. The manifestation of the step in the eroded lingual enamel in the experimental molars was calculated by measuring the distance between the line representing the step and the corresponding level at the enamel–cementum junction (illustrated in Figure 6(b–d)). Measurement data were tabulated and analysed using Statistical Package for the Social Sciences (SPSS) software program, version 22.0 for Windows (SPSS, Chicago, IL, USA). The data were evaluated using one-way analysis of variance (ANOVA) followed by the Tukey *post hoc* test, and independent *t*-tests. *p*-Values <.05 were considered statistically significant.

Results

The behaviour and wellness of the NOD mice

During the 6-week experimental period, daily consumption of both water and acidic drinks was approximately 15 mL per cage. Most of the animals exhibited normal behaviour and

wellness; however, in total 16 mice (15 NOD and 1 wild type) showed signs of illness and were consequently excluded from the study (Figure 1). One mouse from the cola light group was found dead during the experiment, whereas the remaining 15 mice were terminated during the experimental period due to observed abnormal behaviour, sickness, lethargy, and poor general health and appearance. During the experiment, when NOD mice, including those in the control group, showed signs of abnormal behaviour and sickness, we measured serum glucose levels using a Contour XT glucose test kit (Ascensia Diabetes Care, Parsippany, NJ, USA). The results showed high glucose levels exceeding 30 mmol/L. The similar findings, with glucose levels approximately 30 mmol/L, were observed in both NOD control and experimental animals that have survived. By the end of the experiment, the mean weight of the surviving animals was only slightly altered and not significantly different between the groups. Mean weight gain was 0.4 g in both the control and cola drink group, while mean weight loss was 0.5 g and 0.2 g in the sports drink and cola light drink group, respectively. At 9 weeks of age, the salivary flow rate (μL of saliva/100g body weight/20 min) in NOD mice and wild-type mice were not significantly different, while at 17 and 22 weeks of age, salivary flow in NOD mice decreased significantly in all groups, by approximately 35% and 60%, respectively, compared with that in wild type.

The erosive effect of sports drink on the dental enamel in NOD mice

Compared to the control teeth (Figure 2(e–h)), sports drink mandibular molars had rounded cuspal morphology, with

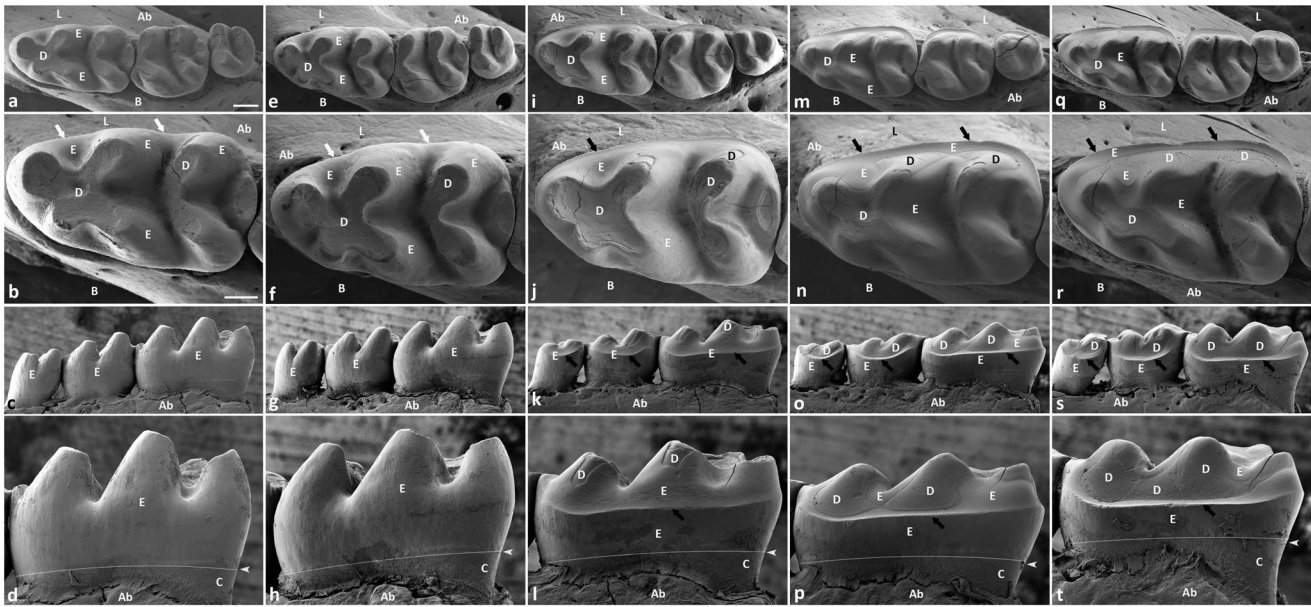


Figure 2. SEM images of mandibular molars from wild type (a–d) and NOD control (e–h) mice, and sports drink (i–l), cola light drink (m–p), and cola drink (q–t) NOD mice. Occlusal view (a, b, e, f, i, j, m, n, q, r) and lingual view (c, d, g, h, k, l, o, p, s, t). (b, f, j, n, r) Higher magnification of occlusal view of mandibular first molar in panels a, e, i, m and q, respectively. (d, h, l, p, t) Higher magnification of lingual view of mandibular first molar in panels c, g, k, o and s, respectively. The white arrows in panels b and f indicate the unaffected and intact lingual surface of the first mandibular wild type and NOD control molar, respectively. The black arrows in panels j, n, r, k, o, s, l, p, and t indicate the affected enamel on the lingual surfaces of the sports drink, cola light drink, and cola drink mandibular NOD molars. The step indicates the border between the unaffected cervical part and the affected occlusal part of the tooth. The white arrowheads and white horizontal lines in panels d, h, l, p and t indicate the enamel–cementum junction. The bar represents 300 μm in panels a, e, i, m, q, c, g, k, o and s, and 200 μm in panels b, f, j, n, r, d, h, l, p and t. E: enamel; D: dentine; C: cementum; Ab: alveolar bone; B: buccal side; L: lingual side.

Table 1. Dimensions of tooth height and enamel thickness.

	Wild-type control	NOD control	NOD Sports drink	NOD Cola light drink	NOD Cola drink
Lingual tooth height	786 \pm 12	792 \pm 19	613 \pm 9*	589 \pm 14*	501 \pm 19*
Buccal tooth height	579 \pm 8	572 \pm 9	529 \pm 13	523 \pm 13	503 \pm 11*
Lingual enamel thickness	67 \pm 3	66 \pm 5	34 \pm 4*	25 \pm 2*	–
Buccal enamel thickness	74 \pm 4	73 \pm 3	75 \pm 2	76 \pm 5	73 \pm 2
Erosive step	–	–	232 \pm 13	211 \pm 15	162 \pm 11

Measured dimensions (mean \pm SD, μm) of mandibular first molar tooth height and enamel thickness are presented. The values represent measurements taken from SEM images of transversely ground plane T1.

(–) Not applicable.

(*) Significant difference, $p < .05$.

evident distinct erosion on the lingual aspect of the teeth (Figure 2(i–l)). The lingual tooth height of first mandibular sports drink molars was 23% lower (613 μm vs. 792 μm) compared to the control (Table 1). The buccal enamel was unaffected; however, the buccal tooth height was reduced by 7% (529 μm vs. 572 μm) (Table 1). The maxillary molars were only slightly eroded, particularly on the lingual mesial half of the tooth (Figure 3(e,f)), while the incisors were unaffected (results not shown).

A recognized erosion pattern, with a distinct step indicating the border between the unaffected cervical and affected occlusal part of the lingual enamel, was observed on all three sports drink mandibular molars (Figures 2(k,l) and 4(i–l)). The eroded enamel covered the dentine at the lingual aspects of the cusps; however, the dentine on cusp L2 and L3 was exposed (Figures 2(l) and 6(b)). The erosive step in the sports drink first mandibular molars, evident at approximately 232 μm as measured from the enamel–cementum junction (Table 1), was continuous on the whole lingual aspect of the tooth (Figures 2(l) and 4(j–l)). Proceeding this

step in an occlusal direction, the enamel was considerably eroded, with complete loss of the superficial prism-free and outer enamel layers (Figure 5(k,l)). The lingual enamel in the sports drink molars, based on the measurements of the transversely ground plane T1, was 48% thinner (34 μm vs. 66 μm) compared to the control molars (Table 1).

Erosive effects of cola light and cola drink on the dental enamel and dentine in NOD mice

In the cola light (Figure 2(m–p)) and cola drink (Figure 2(q–t)) mandibular molars, extensive erosion was observed on the lingual aspect of all three molars. In the cola light first mandibular molars, the lingual enamel was almost completely eroded, and the lingual dentine was exposed. Small areas with remaining enamel were only observed in the pits between cusps L1 and L2 and between L2 and L3 (Figures 2(p) and 4(n–p)). However, in the cola drink molars, the erosive outcome was more extended, and the exposed dentine appeared as a continuous layer (Figures 2(t) and 4(r–t)).

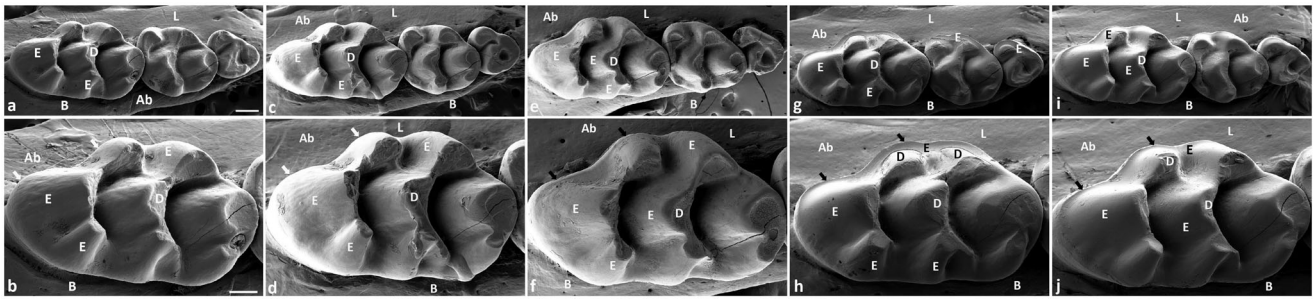


Figure 3. SEM images of maxillary molars from wild type (a, b) and NOD control (c, d) mice, and sports drink (e, f), cola light drink (g, h), and cola drink (i, j) NOD mice. (a, c, e, g, i) Occlusal view of all three maxillary molars from wild type and NOD control mice, and sports drink, cola light drink, and cola drink NOD mice, respectively. (b, d, f, h, j) Higher magnification of occlusal view of maxillary first molar in panels a, c, e, g and i, respectively. The white arrows in panels b and c indicate the unaffected and intact lingual surface of the first maxillary wild type and NOD control molar, respectively. The black arrows in panels f, h and j indicate the affected enamel on the lingual surface of the sports drink, cola light drink, and cola drink first maxillary NOD molars. The step indicates the border between the unaffected cervical part and the affected occlusal part of the tooth. The bar represents 300 μm in panels a, c, e, g and i, and 200 μm in panels b, d, f, h and j. E: enamel; D: dentine; Ab: alveolar bone; B: buccal side; L: lingual side.

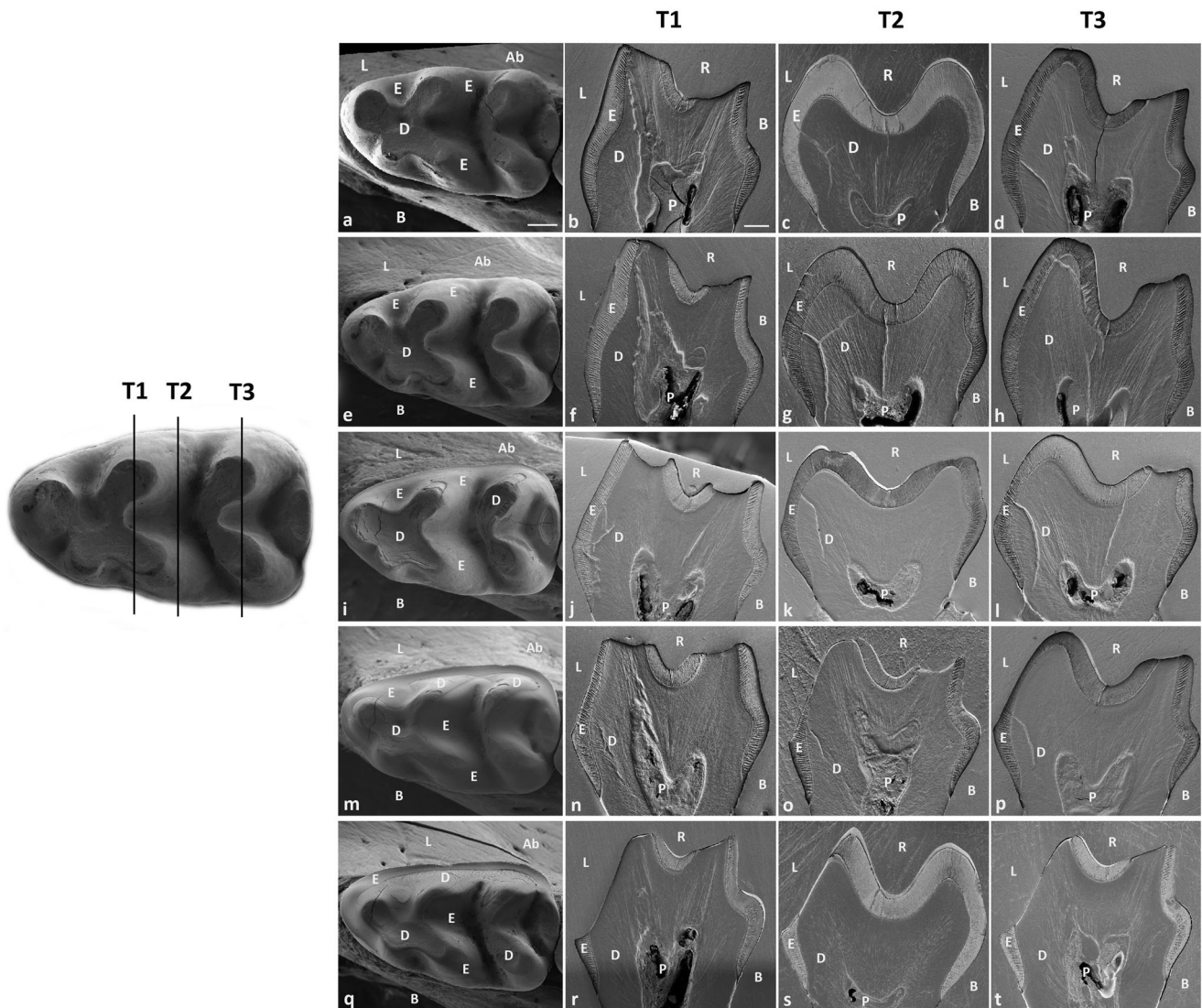


Figure 4. SEM images of transversely ground planes of mandibular first molars from wild type (a–d) and NOD control (e–h) mice, and sports drink (i–l), cola light drink (m–p), and cola drink (q–t) NOD mice. The image on the left shows a representation of a mandibular first molar, indicating the position of the transversely ground planes T1, T2, and T3. In both wild type and NOD control molar, the lingual enamel exhibits full thickness (b–d and f–h). In the sports drink (j–l), cola light drink (n–p), and cola drink (r–t) molars, the lingual enamel exhibits a distinct step with varying degrees of erosion in an occlusal direction. The bar represents 200 μm in panels a, e, i, m and q, and 100 μm in panels b–d, f–h, j–l, n–p and r–t. E: enamel; D: dentine; P: pulp; Ab: alveolar bone; R: resin; B: buccal side; L: lingual side.

In both groups, all three mandibular molars exhibited a typical rounded cuspal appearance with considerable erosion also on the occlusal aspects of the cusps (Figure 2(o,p,s,t)), more pronounced in the cola drink molars (Figure 2(s,t)). Accordingly, on the occlusal aspect of the molars, erosion increased the size of normally obliquing enamel-free areas, which were continuous with the lingual dentine in the cola drink molars and partly continuous in the cola light molars (Figure 2(n,r)). In the cola drink maxillary molars, a marked erosion step on the lingual side was observed on all three

molars (Figure 3(i,j)), whereas in the cola light maxillary molars the erosion step, with large areas of exposed dentine, was even more distinct (Figure 3(g,h)). However, in both cola light and cola group, the erosive effect was more variant in the maxillary molars. Furthermore, the incisors in both groups were unaffected (results not shown).

The transversely ground planes of the cola light and cola drink first mandibular molars showed an erosion pattern that reflected the observations of the sports drink molars; however, there was greater erosive destruction of the lingual

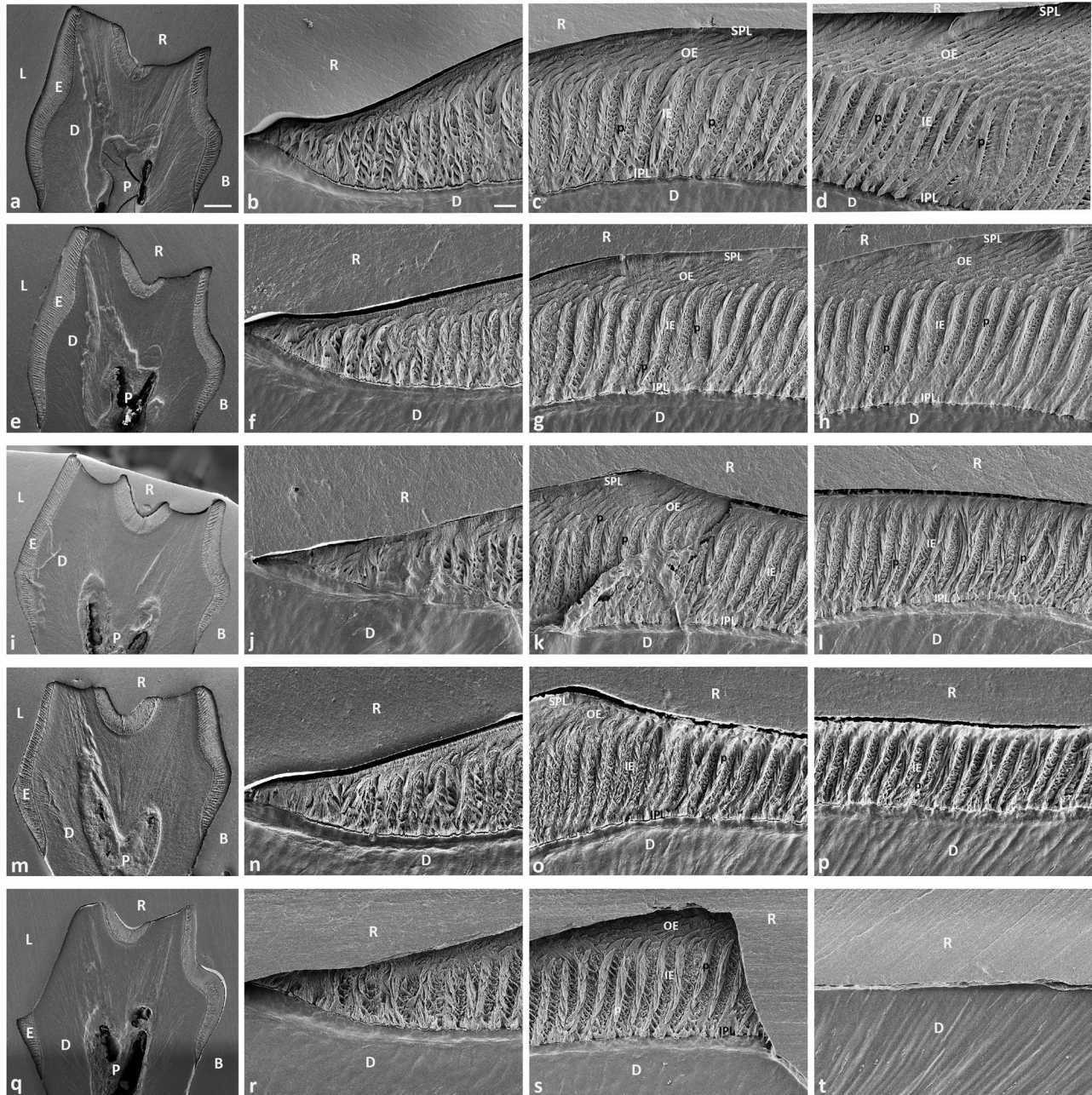


Figure 5. SEM images of lingual mandibular first molar enamel from wild type (a–d) and NOD control (e–h) mice, and sports drink (i–l), cola light drink (m–p), and cola drink (q–t) NOD mice. (b–d, f–h, j–l, n–p, r–t) Higher magnification of lingual enamel from panel a, e, i, m and q, respectively. In both wild type and NOD control molar, the lingual enamel exhibits full thickness and normal basic enamel structure with four layers (b–d and f–h). In the sports drink molar, the lingual enamel exhibits a distinct step indicating the border between the eroded and unaffected enamel (k). An artefact is presented in the middle of panel k due to remnants of organic materials, often appearing in SEM after etching of enamel. The superficial and outer enamel is completely eroded (l). In the cola light drink molar, the enamel is even more eroded, including the partial erosion of inner enamel (p). In the cola drink molar, the lingual enamel shows a distinct step with total erosion of enamel in an occlusal direction (s, t). The bar represents 100 μ m in panels a, e, i, m and q, and 10 μ m in panels b–d, f–h, j–l, n–p and r–t. E: enamel; D: dentine; P: pulp; R: resin; B: buccal side; L: lingual side; IPL: inner prism-free layer; IE: inner enamel; OE: outer enamel; SPL: superficial prism-free layer; p: prism.

enamel (Figures 4 and 5), including significant erosion of the dentine in cola drink molars (Figures 4(r–t) and 5(q)). In both cola light and cola drink mandibular first molars, the lingual and buccal cusps were eroded and reduced in height (Figure 6(c,d)). The lingual tooth height was 26% lower (589 μm vs. 792 μm) in the cola light drink molars and 37% lower (501 μm vs. 792 μm) in cola drink molars compared to the control (Table 1). The enamel on the buccal aspect of the molars was unaffected (Figures 4 and 5); however, the buccal tooth height in the cola light and cola drink molars was reduced by 9% (523 μm vs. 572 μm) and 12% (503 μm vs. 572 μm), respectively (Table 1).

The erosive effects on the cola drink molars were more severe compared to that in the cola light molars, and consequently the erosion of the enamel was more extended in the cervical direction as judged by the presence of the erosive step (Figures 2 and 6). At the transversely ground plane T1, approximately 211 μm , as measured from the enamel–cementum junction, an erosive step was evident in the cola light molars (Figure 6(c); Table 1). In cola drink molars, this step was present at approximately 162 μm from the enamel–cementum junction (Figure 6(d); Table 1). In cola light molars, proceeding from this step in an occlusal direction, the enamel layers were gradually lost, with complete loss of superficial and outer enamel (Figure 5(o,p)). The lingual enamel in cola light molars, based on the measurements on the transversely ground plane T1, was approximately 62% thinner (25 μm vs. 66 μm) compared to the control molars (Table 1). However, in the cola drink molars, the enamel with all its layers was completely eroded occlusally for the erosive step (Figure 4(r–t)). As judged by the lingual outline of the dentine surface at this level, dentine erosion was evident (Figures 4(r–t) and 6(d)).

The pattern of acid-induced dental hard tissue destruction

SEM observations of the morphology (Figure 2) and transversal sections (Figures 4 and 5) of mandibular first molars from both control and all three experimental groups, gave the possibility to study the pattern and consecutive sequence of acid induced dental hard tissue loss (Figure 6). The control molars were not affected (Figure 6(a)), and erosive lesions were evident in increased succession from sports drink (Figure 6(b)), cola light (Figure 6(c)) to cola drink (Figure 6(d)) molars. Accordingly, loss of enamel was initiated at the occlusal half of the tooth, including the tip of the cusps, resulting in reduced enamel thickness and dentine exposure at the tip of the cusps L1 and L2 (Figure 6(b)). The erosive step, marking the border between the affected and unaffected cervical enamel, was evident (Figure 6(b)). The persistent erosion resulted in further loss of dental enamel on cusps L1 and L2 in both the cervical and mesio-distal directions, and simultaneously with the evident initiation of enamel erosion at cusp L3 (Figure 6(c)). At this point, synchronous loss of enamel towards the underlying dentine together with further progression of the erosive step in cervical direction, was observed. Collectively, the enamel

became thinner and the total eroded area above the erosive step was increased (Figure 6(c)). At this stage, coincidental expanding exposure of dentine together with initial erosion of dentine at the top of the cusps was evident; the molars appeared with typical rounded cuspal morphology (Figure 6(c)). At the final stage, almost all enamel above the erosive step, except for some remnants in the pits between the cusps, was destroyed, and the exposed dentine emerged as a continuous layer with progressive erosion in the cervical, mesio-distal, and even pulpal direction (Figure 6(d)). The extensive erosion of dentine in the pulpal direction resulted in loss of the curved outline of the lingual tooth surface (Figure 6(d)).

Discussion

A similar erosion pattern, as described here in NOD mice, has been observed in rats [7–9] and has recently also been described in our study using mice with normal salivary flow [13]. However, the NOD mice showed significantly more erosion. In this study, we aimed to design the experiments and the evaluation method with SEM, similar to the study on mice with normal salivary flow [13], in order to enable a comparison of the results and thereby explore the role of salivary flow rate on development of erosive lesions. However, due to the complexity of NOD mice with regard to when the salivary flow rate decreases, it was not possible to use animals of the same age, i.e. the study was initiated at 7 weeks in normal mice vs. 16 weeks in NOD mice. Therefore, from the time the NOD mice arrived at the animal facility until the beginning of the experiments, and during the 6-week erosive induction (Figure 1), we aimed to minimize attrition by softening the standard laboratory fodder with water. All animals in this study, in both the control and experimental groups, were maintained on the same fodder both before and during the experiment. Importantly, as judged by the tooth morphology at the end of the experiment, the mandibular molars in the control NOD mice (Figure 2(e–h)) did not show increased tooth wear compared neither to that in the wild-type control (Figure 2(a–d)) nor compared to the mice with normal salivary flow in our previous study [13]. Accordingly, we assert that the tooth wear observed in the experimental NOD molars was predominantly due to erosion, and that the comprehensive registration of erosive lesions and their depths in this study are comparable with the findings in mice with normal salivary flow [13].

Different types of acids, amount, lifestyle, and drinking habits and technique have a collected impact on the risk for development of erosive lesions in humans [4,6,32–34]. In this study, the NOD mice were given sports drink (citric acid, pH 3.39), cola light drink (phosphoric acid, pH 3.10), or cola drink (phosphoric acid, pH 2.27) as the only drink for 6 weeks. Therefore, due to these exaggerated conditions, the results have to be carefully extrapolated to humans, in whom the consumption of acidic drinks is more varied and is often combined with other drinks, such as water. In accordance with previous animal studies, the results showed some small individual variations in the erosive effects

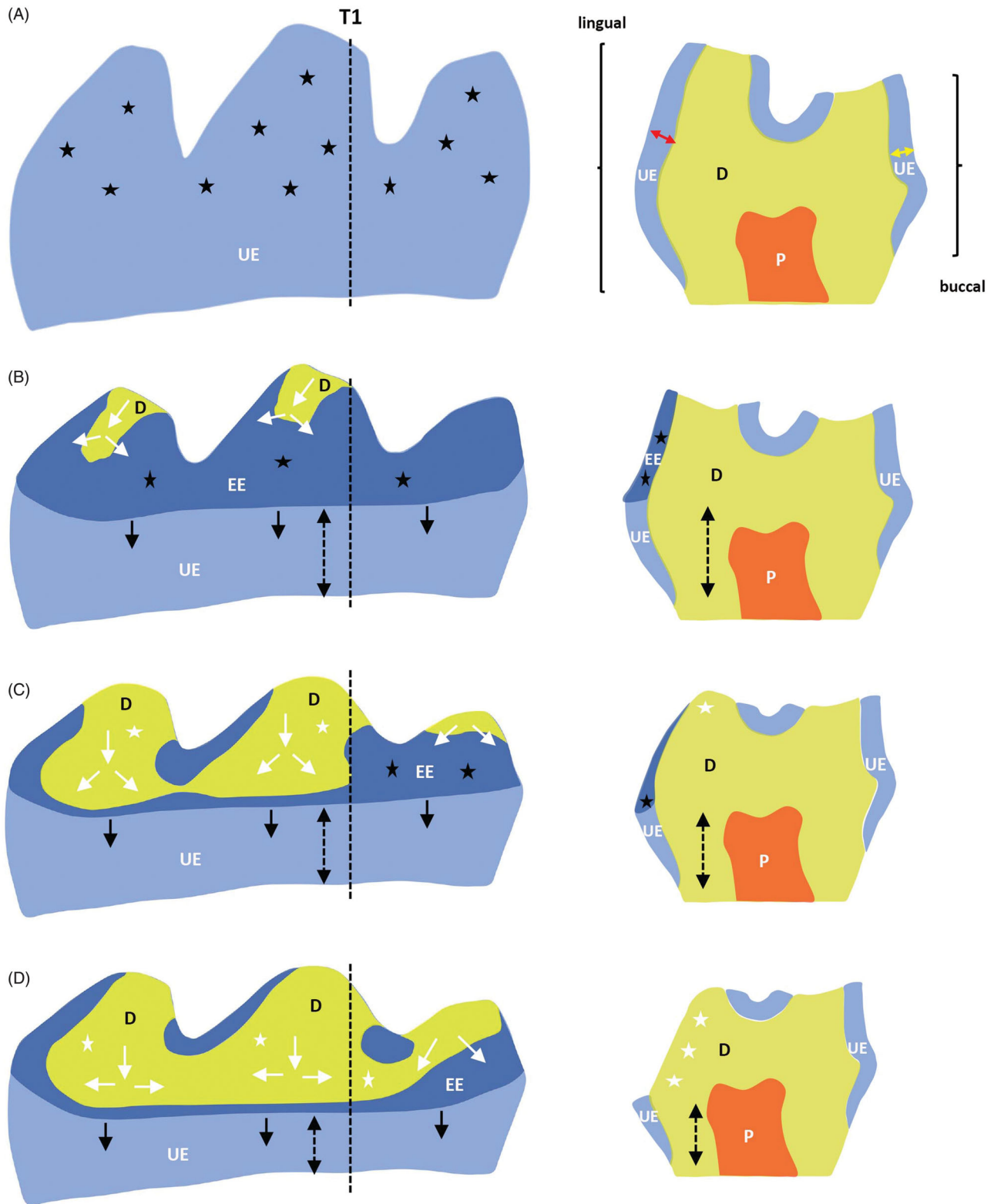


Figure 6. The pattern and consecutive sequence of acid-induced dental hard tissue loss. Schematic representation of lingual view and transversely ground planes (T1) of mandibular first molar from control (A), sports drink (B), cola light drink (C), and cola drink (D) NOD mouse. The stippled vertical line marked T1 indicates the position of the transversal sections presented on right. The black stars in A indicate the part of the tooth at which the enamel erosion will start. The black stars in B and C indicate progression of enamel erosion towards the underlying dentine. The white stars in C and D indicate progression of erosion into dentine and towards pulpal direction; white arrows represent the direction of cervical and mesio-distal erosion. The black arrows show the progression of erosion and movement of the erosive step in the cervical direction; the stippled black arrows indicate the distance between the step and enamel-cementum junction. UE: unaffected enamel; EE: eroded enamel; D: dentine; P: pulp.

between mice within the same experimental group, which may be due to the differing frequency and amount of drink consumed, which was not possible to control during the experiment. However, the overall consumption of drinks was not significantly different between the groups. In general, the findings show that all three acidic drinks resulted in higher erosive tooth destruction of NOD molars compared with that in mice with normal salivary flow [13]. Despite this, no erosive influence on the incisor teeth was observed in the groups. It appears that the protective property of yellow-brown iron pigmentation on the labial enamel of mouse incisors, which is more resistant to acid [35], was decisive in this outcome. As previously reported in clinical [4,36] and animal [13] studies, NOD mouse maxillary molars are considerably less affected compared to mandibular molars (Figures 2 and 3), probably due to anatomical relations where the acid is present for a longer time in the sublingual compared to palatal area of the oral cavity. However, it is evident that the reduced salivary flow rate has an impact on the development of erosive lesions in the maxillary molars. Compared to the findings in mice with normal salivary flow rates [13], NOD mouse maxillary molars had a marked erosion step on the lingual side of all three molars in the cola and cola light drink groups (Figure 3). However, the reason that maxillary molars in cola light group were more affected compared to that in the cola group, which in general exhibited more severe lesions, is uncertain.

The progression of dental erosion is influenced by both physical factors (temperature, salivary flow rate), and chemical factors (degree of saturation, presence of inhibitors, type of acid, buffering, pH, fluoride) [33]. The association between low salivary flow rate and the occurrence of dental erosive lesions has been investigated in a few studies only [11,18–21]. Regarding the acidic drinks, this study and the previous study [13] confirms that several factors are involved in the complicated chemistry of dental erosion, such as type of acid, low acid dissociation constant (pKa), and low pH. These factors influence the development and severity of dental erosion in mouse molars. Erosive lesions were evident in increasing succession from sports drink (Figure 6(b)), cola light (Figure 6(c)) to cola drink (Figure 6(d)) molars. In all experimental NOD mice groups, acidic drinks exhibited higher erosive potential compared to corresponding groups in the mice with normal salivary flow [13]. It was also evident that the erosive step in the experimental NOD molars was extended significantly further in the cervical direction compared with that in the mice with the normal salivary function. However, the most cervical part of the experimental NOD mouse lingual enamel was not affected. The same erosive pattern has been shown in rat [8] and mouse [13] molars. Since the enamel is not gradually eroded along the whole lingual surface that is exposed to acid, it may be assumed that the gingiva covering the cervical part serves as protection.

In this study, the transversal ground planes of mouse molars enabled detailed measurements of tooth erosion. As previously discussed [13], achieving an ideal transversal ground plane of mouse molars through the correct cusps is

technically difficult due to their small size, which may have affected the accuracy of the measurements. However, it was considered unlikely that this slight variation would mask the significant differences in enamel thickness after erosion between the groups. This is reflected by the enamel thickness measurements in the wild-type mice and NOD control mice in this study, and NOD control mice from previous study [13] using the same method, which showed accurate and similar values in both (Table 1).

NOD mice were selected in this study, since they represent a well-known model of Sjögren's syndrome with documented progressive loss of salivary gland function and significantly reduced salivary flow rate between week 17 and 24 [28,29]. In order to investigate the impact of decreased salivary flow rate on development of erosive lesions, mice at about this age were used, with significantly reduced salivary flow compared to wild type, measured at both 17 and 22 weeks of age. However, challenges during the study were experienced; 15 NOD mice died during the experiment (Figure 1). According to the animal supplier Taconic Biosciences, the present mouse model exhibits destructive autoimmune pancreatic insulinitis as early as 4 weeks of age, and insulin-dependent diabetes is found in some females beginning at 12 weeks of age and in approximately 50% of females by 24 weeks of age. Other studies have also shown that NOD mice spontaneously develop diabetes, the progression of which is similar to that of human type 1 diabetes [37,38]. Interestingly, type 1 diabetes incidence in NOD mice and the rate of disease progression are affected by the gut microflora and the drinking water pH, which also affects the composition and diversity of commensal bacteria in the gut [39]. It has been shown that female NOD mice that were maintained on acidic water developed more frequently insulinitis and hyperglycaemia compared with those on neutral pH water [39]. Accordingly, it is likely that some of the NOD mice that were exposed to acidic drinks in our study developed diabetes with lethal outcome. However, it was also experienced that two mice from the control group, maintained only on distilled water, died (Figure 1). Even though, in NOD mice, the common clinical symptoms of diabetes are present as in humans, the mice have larger resistance to development of ketoacidosis. In consequence, they can remain alive approximately 2–4 weeks after the onset of disease without treatment with insulin. Finally, if not treated, death results from dehydration, rather than ketoacidosis [40,41]. Since the onset of salivary gland infiltration and salivary flow reduction in NOD mice has been shown to be independent of blood glucose status [42], we assume that the salivary flow in this study was not influenced by poorly regulated diabetes. Further, the salivary flow rate, as measured in this study, decreased as previously shown [28] with age, and was not influenced by diet since it was similarly reduced in all NOD mice. Collectively, it was assumed that sick animals that were excluded from the study developed diabetes either spontaneously or due to consumption of acidic and sugar containing drinks. However, although treatment of overtly diabetic NOD mice is possible [43], in this study those animals were terminated.

There are several studies looking at a possible association between the presence of dental erosions and saliva. However, most of those have been *in vitro* and *in situ*. Considering guiding principles underpinning the humane use of animals in scientific research, the three Rs, we did not find it reasonable to include more than approximately 10 NOD mice, prone to develop diabetes, in each group. This study is the first *in vivo* experiment that highlights the role of saliva in development of dental erosion. Use of animal models, and especially mice, when studying oral pathology in humans is widely accepted. In this study, a standardized *in vivo* model was used, which is widely described in our previous paper [13] and has been shown to be suitable for studying even initial erosive lesions. In addition, our SEM images as shown previously [13] and in this study show the same pattern of erosion on mouse molars as in the humans, with the typical cuppings/pits on the occlusal surface of the molars [36].

In conclusion, animal models are highly advantageous due to the salivary influence and soft tissue interactions that resemble the human oral environment. NOD mice, exhibiting low salivary flow rate at a certain age, may serve as a beneficial model for studying reduced saliva as a risk factor for dental erosive wear, however, the frequent development of diabetes may be challenging. Erosive lesions appearing in increased succession from sports drink, cola light to cola drink molars indicated that chemical factors such as the low pH and type of acid are important factors for development of dental erosive lesions. It may be concluded that the severity of the erosive lesions increases when an important protective factor, such as saliva, is reduced or missing. In addition to salivary flow rate, the composition of saliva may be important factor having impact on its protective properties. Different fluoride treatments should also be explored as part of prophylactic treatment for individuals at risk for dental erosion. These aspects may be important to address in future studies using similar animal model and experimental method.

Disclosure statement

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