UiT The Arctic University of Norway

Faculty of Health Sciences, Department of Clinical Dentistry

Molar-Incisor Hypomineralization (MIH)

Prevalence among 16-year-old adolescents: A case-control study of children with a low Apgar score at birth and a study on tooth formation and antibiotics in mice

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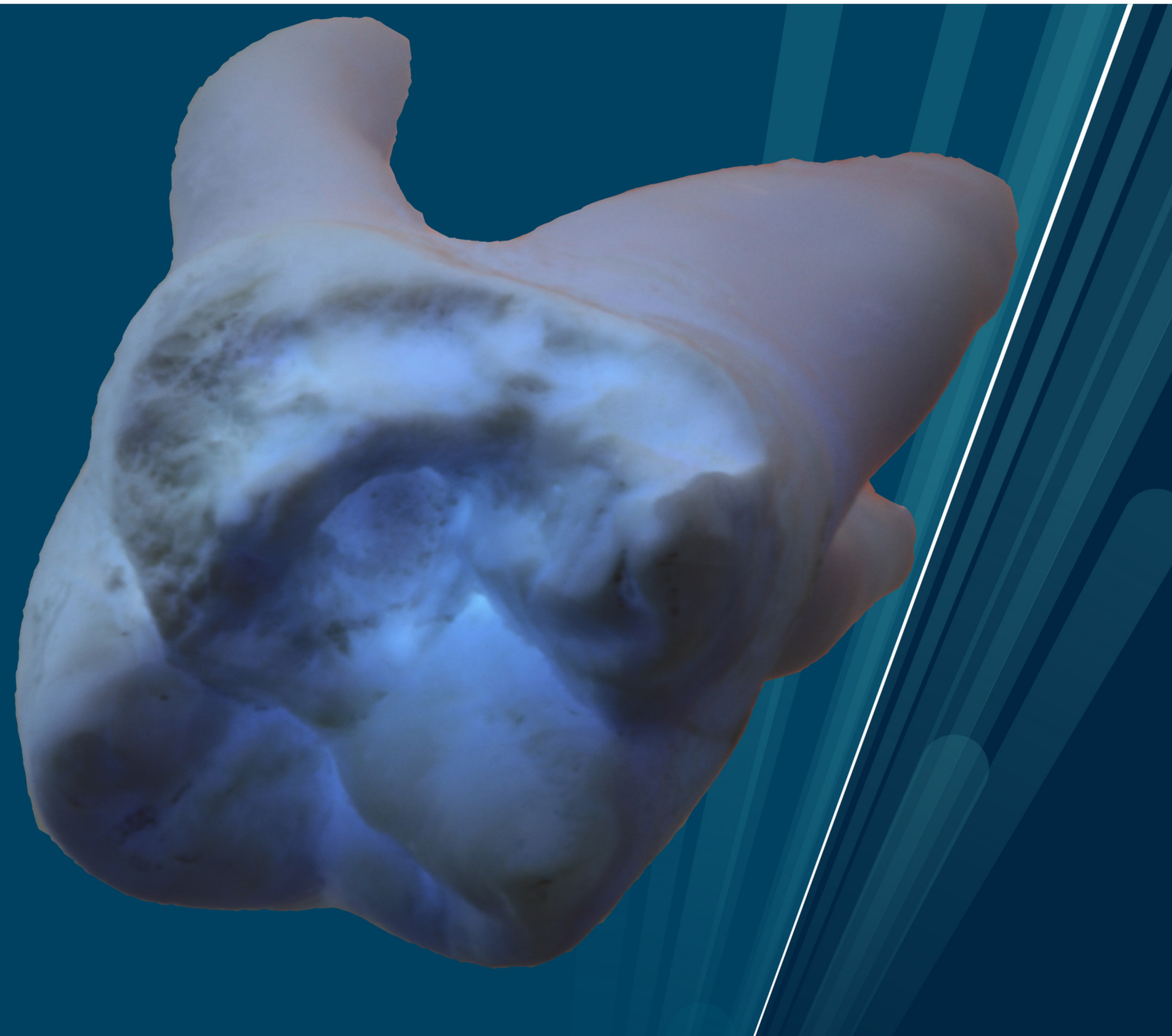

P*5

To Juliane

Contents

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Abbreviations

AI:	Amelogenesis imperfecta
ANOVA:	Analysis of variance
ARRIVE:	Animal Research: Reporting of In Vivo Experiments
BMP:	Dental behavior management problem
BPA:	Bisphenol A
CI:	Confidence interval
DEJ:	Dentin-enamel junction
DFA:	Dental fear and anxiety
E11:	Embryonic day 11
EAPD:	European Academy of Paediatric Dentistry
ENAM:	Enamelin
FDI:	Federation Dentaire International
FOTS:	Norwegian food safety authority
FPM:	First permanent molar
HIF1-α:	Hypoxia-inducible factor 1-α
HSPM:	Hypomineralized second primary molar
KLK4:	Kallikrein-related peptidase 4
mDDE:	Modified developmental defects of the enamel
micro-CT:	X-ray microtomography
MIH:	Molar-Incisor Hypomineralization
MMP20:	Matrix metallopeptidase 20
P1:	Postnatal day one
PCB:	Polychlorinated biphenyls
PDHS:	Public Dental Health Service
PEB:	Posteruptive breakdown
qBSE:	Quantitative backscattered electron
SEM:	Scanning electron microscopy
REK:	Regional Committee of Medical and Health Research Ethics
SD:	Standard deviation
TRPV1:	Transient receptor potential ion channel
UiT:	Arctic University of Norway
VDR:	Vitamin D receptor

List of papers

This thesis is based on the following three papers, referred to in the text by their corresponding Roman numerals.

Paper I

Schmalfuss A, Stenhagen KR, Tveit AB, Crossner CG, Espelid I.

Canines are affected in 16-year-olds with molar-incisor hypomineralisation (MIH): an epidemiological study based on the Tromsø study: "Fit Futures".

Eur Arch Paediatr Dent. 2016;17(2):107-113.

doi:10.1007/s40368-015-0216-6

Paper II

Sidaly R, **Schmalfuss A**, Skaare AB, Sehic A, Stiris T, Espelid I.

Five-minute Apgar score≤5 and Molar Incisor Hypomineralisation (MIH) - a case control study.

BMC Oral Health. 2016;17(1):25.

doi:10.1186/s12903-016-0253-5

Paper III

Schmalfuss AJ, Sehic A, Brusevold IJ.

Effects of antibiotics on the developing enamel in neonatal mice [published online ahead of print, 2021 Oct 29].

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Abstract

Background/Aims: Molar-Incisor Hypomineralization (MIH) is one of the most common dental developmental disorders. The global prevalence if MIH is estimated to be approximately 13%. The etiology of MIH is still elusive. Suspected etiological factors are prenatal, perinatal or early-life illnesses or events; several medications; environmental toxins; and genetic factors. The evidence is weak or absent for most of these factors. The overall aim of this thesis was to assess the MIH prevalence, distribution of affected teeth and MIH severity in northern Norway and to examine a possible association between birth asphyxia and MIH. Additionally, the effects of gentamycin and ampicillin on the developing enamel in neonatal mice in vivo were investigated.

Methods: To assess the MIH prevalence, the distribution of affected teeth and MIH severity, a cross-sectional health survey including 16-year-olds was performed. The diagnosis of MIH was based on clinical photographs. To examine the association between birth asphyxia (Apgar score ≤ 5 after 5 min) and MIH, a cross-sectional, case-control study of 8- to 10-year-old children was performed. The diagnosis of MIH was based on clinical examination and photographs. The effects of gentamycin and ampicillin on the developing enamel were assessed in neonatal mice treated with intravenous injections of these antibiotics for 4 days. X-ray microtomography (micro-CT) was used to analyze the mineral density (MD) of the enamel and the proportion of the enamel object volume (vol%) in first molars and incisors.

Results: The prevalence of MIH in northern Norway was 13.9%. Maxillary molars were 1.6 times more frequently affected than mandibular molars. Affected incisors were recorded in 41.8% and affected canines in 22.8% of the participants with MIH. Only opacities were reported in 54% of the affected molars, while the other MIH features were more severe. The prevalence of MIH did not differ between the children born with an Apgar score ≤ 5 registered 5 min after delivery and the control group. Regarding the effect of antibiotics on developing enamel in neonatal mice, the analysis showed significantly lower vol% in maxillary and mandibular molars in the study group than in the control group. A lower vol% and lower MD of the enamel in most segments of incisors were observed in treated mice than in control mice.

Conclusions: The prevalence of MIH (13.9%) as well as the distribution pattern and severity of affection were consistent with previous Scandinavian reports. Approximately one-quarter of all participants affected by MIH had at least one affected canine. An Apgar score ≤ 5 at 5 min after delivery did not increase the incidence of MIH. Intervention with high-dose antibiotics given to neonatal mice influenced the development of molars and incisors. The analysis of teeth in neonatal mice with micro-CT could be a valid model for further research on MIH.

1. Introduction

1.1. Molar-Incisor Hypomineralization (MIH)

In the late 1970s, an increasing number of children with idiopathic enamel hypomineralization of incisors and first permanent molars (FPMs) were reported within the Public Dental Services in Sweden. In 1987, Koch et al. (1987) described this condition for the first time, and the description was consistent with the current definition of Molar-Incisor Hypomineralization (MIH). Several different terms for this condition were used during the following years by clinicians and researchers. Initiated by the arising confusion with the use of different definitions of MIH in several presentations and publications, it was deemed urgent to clearly define the term MIH. A proposed definition of the term MIH was published subsequent to the 5th Congress of the European Academy of Paediatric Dentistry (EAPD) in 2000 (Weerheijm et al., 2001), and since 2001, it has been the generally accepted term.

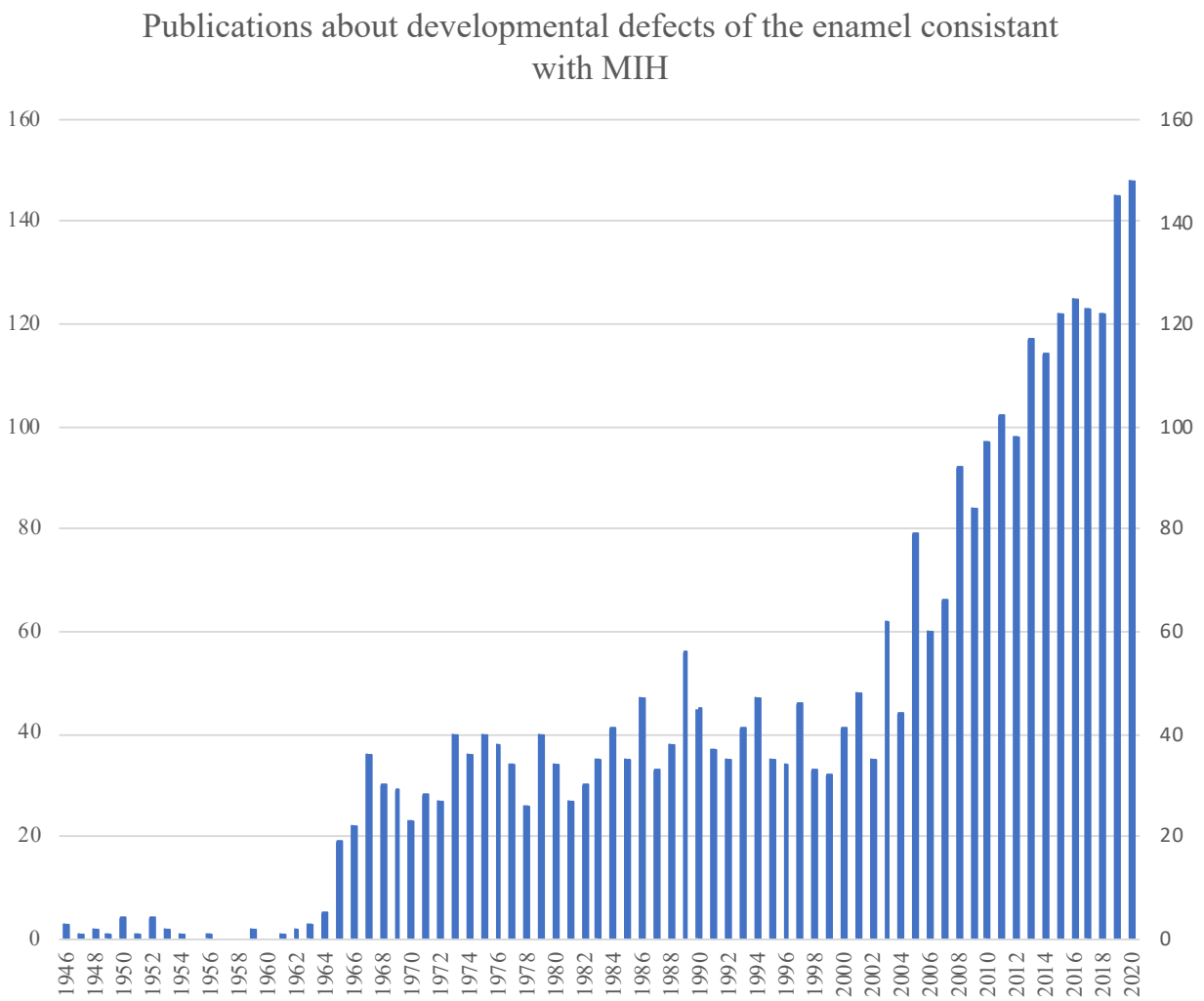
MIH is one of the most prevalent dental developmental aberrations of systemic origin and has asymmetric distribution in the dentition (Weerheijm et al., 2001). Following the EAPD criteria, MIH is now defined as 'demarcated, qualitative developmental defects of the enamel of one or more FPMs with or without the affection of permanent incisors and canines' (Lygidakis et al., 2010, Weerheijm et al., 2003).

The global prevalence is assumed to be 13.1% (Elhennawy et al., 2018). In 2015, it was estimated that approximately 878 million people in the world were affected by MIH, with 17.5 million new cases each year (Schwendicke et al., 2018). The prevalence of MIH seems to vary considerably among countries as well as regions, the reason for which is not apparent.

The exact etiology of MIH remains unclear despite intensified research activity in recent decades (Figure 1). However, prenatal, perinatal or early-life illnesses or events; several medicines; environmental toxins; and genetic factors are suspected to be associated etiological factors (Dantas-Neta et al., 2018, Silva et al., 2016, Teixeira et al., 2018). The evidence is weak or absent for most of these factors, and it is assumed that the etiology is multifactorial (Serna et al., 2016, Silva et al., 2016).

1.1.1. Indices for MIH used in the past and proposed for the future

Developmental defects of the enamel are described as demarcated or diffuse opacities or hypoplasias or both. These defects are usually classified in line with the index for modified developmental defects of the enamel (mDDE) of the Federation Dentaire International (FDI) (FDI, 1992). Concerning MIH, the mDDE index has strong deficiencies. Using the mDDE index, hypomineralizations due to MIH, dental fluorosis or other enamel defects cannot be differentiated (Weerheijm et al., 2003). To make precise distinctions between posteruptive breakdown (PEB), a prominent feature in MIH, and enamel hypoplasia, the mDDE index is not considered to be



Figur 1: Number of publications per year about developmental defects of the enamel consistent with MIH. The data were conducted using specific search terms (Table 1) in the PubMed databases from the year 1946 to 2020. A distinct increase is shown especially in the years after the enamel defect was termed MIH by the EAPD in 2001 (Weerheijm et al., 2001).

Search	Search Query in PubMed	Items found
#3	#1 AND #2	2924
#2	"humans"[All Fields] OR "human"[All Fields] AND "humans"[MeSH Terms].	18981428
#1	"dental enamel hypoplasia"[MeSH Terms] OR ("dental"[All Fields] AND "enamel"[All Fields] AND "hypoplasia"[All Fields]) OR "dental enamel hypoplasia"[All Fields] OR ("molar"[All Fields] AND "incisor"[All Fields] AND "hypomineralization"[All Fields]) OR "molar incisor hypomineralization"[All Fields].	3350

Table 1: Search Query in PubMed. By using the key words "molar, incisor and hypomineralization", a systematic search in the PubMed database was conducted, where both free and MeSH terms were used. Animal research was excluded in this search. The number of articles published each year from 1946 to 2020 is presented (Figure 1).

adequate. In addition, treatments of atypical caries and restorations as well as extractions due to MIH are not included in the mDDE index (Jalevik et al., 2018).

Several terms for the clinical description of this phenomenon, such as “idiopathic enamel hypomineralization”, “nonfluoride hypomineralization in permanent molars”, “cheese molars”, and “Morbus S”, were initially used (Koch et al., 1987, van Amerongen and Kreulen, 1995, Weerheijm et al., 2001). In 2001, MIH was defined by the EAPD as a hypomineralization of systemic origin of one to four FPMs, frequently associated with affected incisors. The clinical description of MIH-affected teeth ranges from white, yellow or brown demarcated opacities to severely affected enamel with PEB. The clinical description of MIH-affected teeth also includes atypical restorations as well as extracted molars due to MIH (Weerheijm et al., 2001).

Revised editions of the EAPD criteria for MIH with several clarifications were published in 2003 and 2010 (Weerheijm et al., 2003, Lygidakis et al., 2010). It was then concluded that secondary primary molars as well as the tips of the permanent canines could be affected, in addition to FPMs and incisors. Furthermore, it was recommended that defects less than 1 mm should not be reported (Lygidakis et al., 2010). The severity of teeth affected by MIH is classified as mild or severe. However, the hypersensitivity of affected teeth and the need for treatment of the affected tooth are not included in the evaluation of the severity. The utilization of internationally standardized diagnostic criteria would make studies of MIH prevalence and treatment outcomes comparable.

Several new MIH indices have been published in the last decade. These indices focus on a more detailed description of the location and extent of opacities, PEB and atypical restorations due to MIH. Additionally, possible problems with hypersensitivity as well as the risk for further decay and increasing symptoms due to hypersensitivity should be evaluated (Steffen et al., 2017, Oliver et al., 2014, Cabral et al., 2020). The main intention of a new MIH index is to obtain an exact description of the effects and to offer dental practitioners help with a standardized approach for dental treatment of MIH (Steffen et al., 2017).

1.1.2. The principles of disturbances leading to MIH

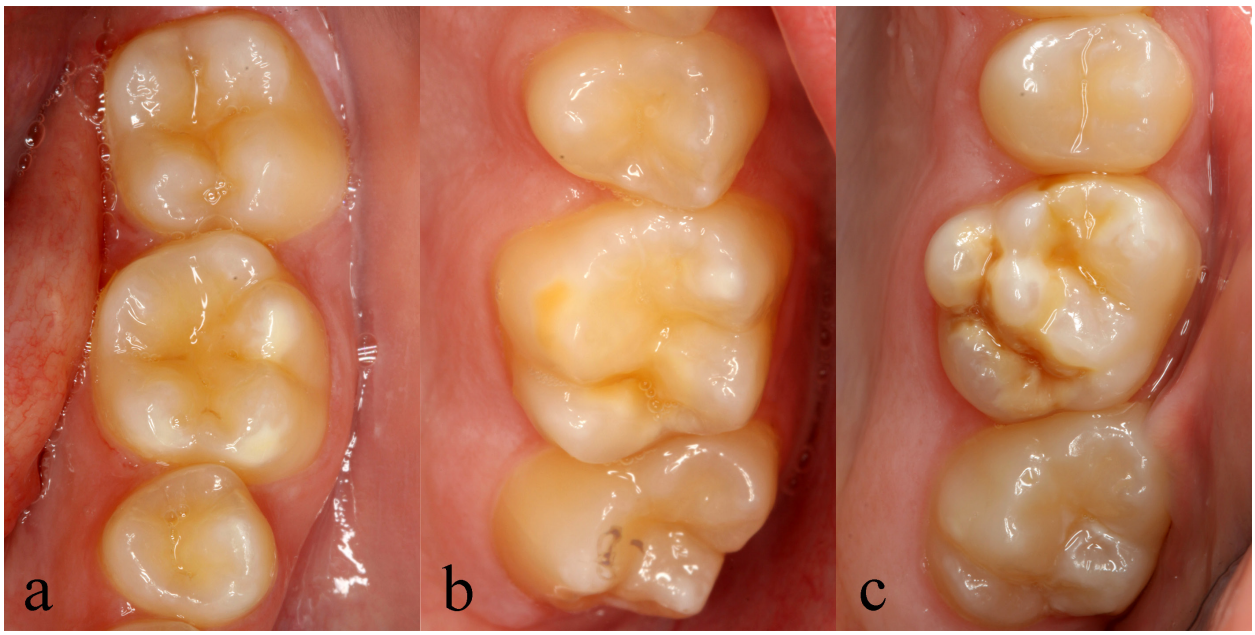
The causative factors for MIH are still uncertain, and the biological process leading to the disruption of amelogenesis is not known. It is accepted that MIH is caused by a disturbance in amelogenesis within the phase of the secretion of enamel matrix proteins, the following short transitional phase or the maturation phase. The time interval for interferences in amelogenesis related to MIH extends from the prenatal period to early childhood. The ameloblasts are sensitive to both direct and indirect insults during the complex process of amelogenesis. Basic research demonstrates that ameloblasts are highly susceptible to even minor changes in their environment, such as temperature increases, hypocalcemia and changes in pH levels (Sui et al., 2003, Tung et al., 2006, Yamaguti et al., 2005). Such conditions might be experienced in relation to not only birth but also early childhood.

1.1.3. MIH prevalence in a global view

The mean (95% CI) global prevalence of MIH is 13.1% (11.8-14.5%), with a significant difference among regions and countries (Elhennawy et al., 2018). The prevalence of MIH varies from 1.2% in New Delhi, India (Goswami et al., 2019), to 40.2% in Rio de Janeiro, Brazil (Soviero et al., 2009). The highest mean prevalence at the country level is reported for Spain (21.1%; 17.7-24.7) and at the continent level for South America (18.0%; 13.8-22.2), and the lowest mean prevalence is reported for Africa (10.9%; 4.2-17.6), while the prevalence is moderate in Europe (14.3%; 12.2-16.3) (Zhao et al., 2018). In Nordic countries, the prevalence ranges from 12.2% in Sweden (Jalevik et al., 2018), 13.9% in Norway (Schmalfuss et al., 2016), and 17.0% in Finland (Alaluusua et al., 1996b) to 37.3% in Denmark (Wogelius et al., 2008). Additionally, within different regions in Germany or different age groups in Sweden, wide ranges of prevalence rates, from 4.3% to 14.6% and 4.4% to 15.4%, respectively, have been reported (Petrou et al., 2013, Koch et al., 1987). The variations in the prevalence of MIH may be explained by ethnic variations, environmental differences, the use of different diagnostic criteria and the examination of different age groups (Schmalfuss et al., 2016, Vieira, 2019b, Weerheijm et al., 2001).

1.1.4. MIH-affected teeth (severity and distribution)

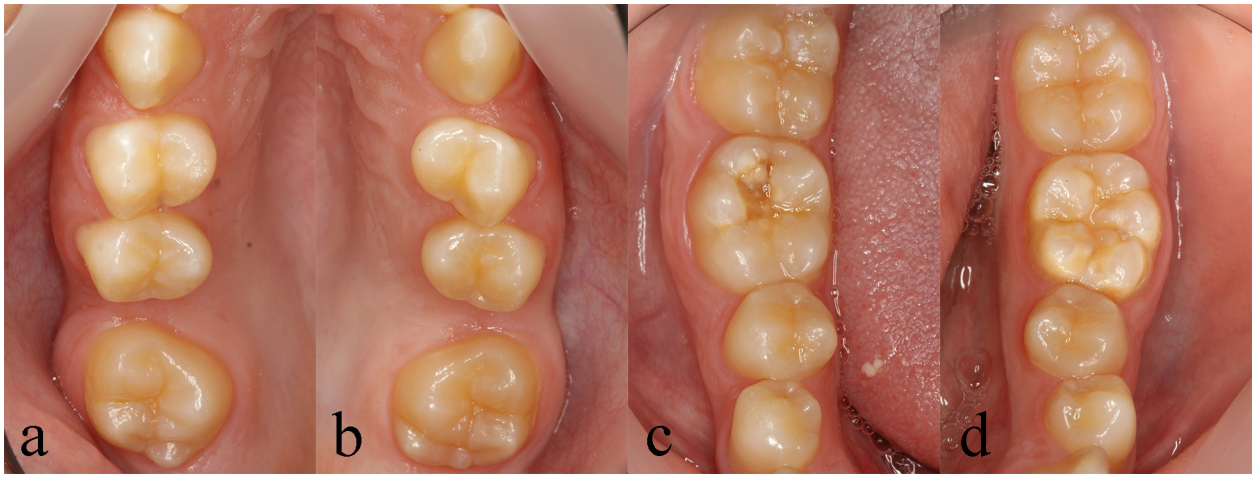
Opacities of the enamel are the primary characteristics of FPMs and incisors affected by MIH. The affected enamel is described as clearly demarcated and variable in size and shows an alteration of translucency, with a color varying from white to yellow or brown (Figure 2) (Jalevik and Noren, 2000, Weerheijm, 2004). Most opacities extend to less than one-third of the tooth surface. Due to the porosity of the affected enamel, parts of the enamel can easily chip off after eruption, usually due to masticatory forces. These defects are referred to in the literature as PEB, which affects approximately one-fifth of molars diagnosed with MIH. These defects are most frequently located on occlusal surfaces due to the enamel's reduced physical properties and natural bite pressure. PEB is less frequent in affected incisors (Petrou et al., 2015). The restoration of affected enamel, usually due to PEB or atypical caries, is termed atypical restoration. These restorations are often distinct from other restorative treatments due to their size, shape or location. Atypical caries are often located on cusps, extending to the buccal and palatal or lingual surfaces, which is an atypical location for cavities due to caries (Oliver et al., 2014). In addition, opacities are frequently seen at the margin of atypical restorations. It is believed that most teeth affected by MIH, even opacities camouflaged by direct restorations, can be detected. Prosthetic crowns are occasionally used for the restoration of FPMs with severe enamel defects. This treatment is mainly done in adults and rarely in children or adolescents (Zagdwon et al., 2003). An exception is stainless-steel crowns, which are an approved temporary treatment for severely affected FPMs in children and adults (Zagdwon et al., 2003).



Figur 2: Examples of affected first permanent molars from three participants with MIH. Defects vary in color, from white (a) and cream (b) to brown (c).

In cases when an FPM is extracted (Figure 3), it is difficult to know whether the FPM was extracted due to MIH or a different diagnosis. The extraction of FPMs due to MIH is an accepted solution in cases of severely affected FPMs (Eichenberger et al., 2015, Jalevik and Moller, 2007). In any such case, the persisting FPMs and incisors should be examined to assess whether the tooth has been removed due to MIH. In addition, considerable information can be obtained from dental records available from dental services as well as patient interviews.

The variable clinical presentation, leading to enormous heterogeneity in published data, is a typical feature of MIH. The number and location of affected teeth as well as the location of



Figur 3: Participant with extracted first permanent molars due to MIH in the upper jaw (a & b) and first permanent molars affected by MIH in the mandible (c & d). Tooth 36 is mildly affected and shows an opacity (d). Tooth 46 is severe affected, showing an opacity and posteruptive breakdown of the enamel (c).

affected enamel on the tooth surface are usually asymmetrically distributed. Approximately 2.8 ± 1.7 teeth are affected in each individual with MIH (Petrou et al., 2013), while the mean number of hypomineralized FPMs is reported to be 2.0 ± 1.1 (Petrou et al., 2015). The proportion of individuals with only one affected FPM varies from 10.4% (Elzein et al., 2019) to 39.2% (Petrou et al., 2015). A positive correlation between the number of affected teeth and the severity of MIH has been reported (Petrou et al., 2013).

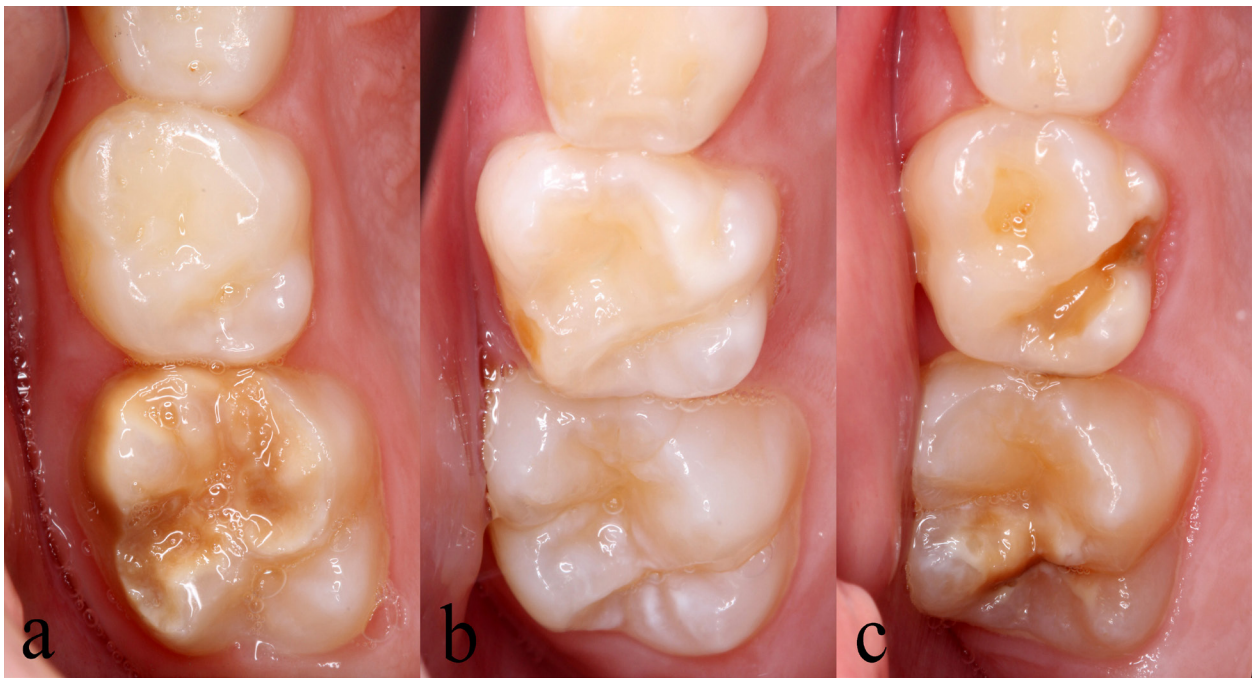
MIH defects are described on permanent cuspids as well. A Finnish study showed that 10.7% of affected patients had at least one affected canine (Tseveenjav et al., 2020), while another study showed that 19.3% of all participants diagnosed with MIH had one or more affected canines (Dietrich et al., 2003).

Conflicting results are presented regarding the susceptibility of upper and lower, as well as right and left arch, FPMs (Elzein et al., 2019, Vieira and Manton, 2019). Most studies show that maxillary permanent incisors are more frequently affected than mandibular incisors (Lygidakis et al., 2008a, Cho et al., 2008). A typical clinical feature of MIH is the asymmetrical presence and severity of MIH lesions, which contrasts with most other enamel hypoplasias and hypomaturations (Biondi et al., 2019).

Several approaches exist to classify the severity of MIH. The most common is the rating of Lygidakis et al. (2010) distinguishing between mild and severe affection. In this rating, opacities without PEB and atypical restorations are labeled mild cases, while all other cases, including those with hypersensitive teeth, are labeled severe. Several other more complex scoring systems of MIH have been published, focusing on the extension of enamel defects, the number of affected teeth, and symptoms caused by affected teeth, to yield a more exact description of the severity. These scoring systems are both tooth- and patient-based (Ghanim et al., 2015, Cabral et al., 2020). Other MIH indices are designed to describe the need for treatment of the affected patient (Steffen et al., 2017).

1.1.5. Hypomineralized second primary molar (HSPM)

Second primary molars are reported to be affected by MIH, similar to FPMs (Figure 4) (Sidhu et al., 2020). This condition is termed hypomineralized second primary molar (HSPM) in the literature (Elfrink et al., 2008, Elfrink et al., 2009), and the reported prevalence ranges from 0-21.8% (Elfrink et al., 2015). Similar to MIH in FPMs, the etiology of HSPM remains unclear (Serna Muñoz et al., 2020). Since the period of enamel maturation for second primary molars and FPMs coincides, it is speculated that HSPM and MIH have the same etiology (Elfrink et al., 2012, Butler, 1967, da Silva Figueiredo Se et al., 2017). The presence of HSPM seems to be a clinical predictive factor for MIH (Taylor, 2017, Temilola et al., 2015), and children with HSPM have an approximately 4.7 times higher risk of being diagnosed with MIH (Garot et al., 2018).



Figur 4: Examples of affected second primary molars and first permanent molars from three participants with MIH (a&c) and HSPMs (b&c). Defects vary in severity from diffuse yellow opacities in teeth 16 (c) and 55 (b) to more severe changes such as PEB in teeth 16 (a) and 55 (c).

1.1.6. MIH - histological, chemical and mechanical properties

The histological, chemical and mechanical properties of hypomineralized enamel due to MIH are in clear contrast to those of sound enamel (Farah et al., 2010b, Elfrink et al., 2012). Concomitantly, the properties of MIH-affected enamel are highly variable both within and between lesions (Crombie et al., 2013). Affected, hypomineralized enamel can be distinguished from sound enamel visually due to its opacity and color, varying from white and cream to brown. In general, all surfaces of the crown can be affected, while the cervical regions are typically spared (Crombie et al., 2013). In affected FPMs, the occlusal surface is most often affected, followed by the buccal surface. The buccal surface of the crown is predominantly affected in incisors (Petrou et al., 2015). A possible explanation of the phenomenon that the occlusal and buccal surfaces are more often affected is their increased thicknesses compared with those of the lingual, palatal, and cervical regions of enamel (Lygidakis et al., 2010, Jalevik and Noren, 2000).

The characteristic opacity and color of hypomineralized enamel is caused by less organized enamel prisms as well as larger interprismatic spaces. Usually, enamel from the surface to the enamel-dentin junction is affected (Mahoney et al., 2004). Affected enamel shows an increased porosity percentage, ranging from 5 to 25%, compared with sound enamel (Crombie et al., 2013). The higher the degree of opaqueness, the higher the porosity percentage (Xie et al., 2007). Creamy or brown opacities have a higher content of serum proteins such as albumin, an inhibitor of crystal growth, leading to more porous enamel and worse mechanical properties, than white opacities (Da

Costa-Silva et al., 2011, Farah et al., 2010a). Hypomineralized enamel has a 3-15 times higher protein content than normal enamel (Mangum et al., 2010). Analyses of the microstructure show a well-demarcated border between affected and normal enamel (Fagrell et al., 2010). Nevertheless, enamel adjacent to a clinically visible opacity seems to have lower mechanical properties as well (Chan et al., 2010). This fact is important to keep in mind for the clinical assessment of MIH-affected teeth.

Regarding chemical properties, affected enamel shows a significant decrease in mineral content, primarily calcium and phosphorus, while an increase in carbon content is shown compared with nonaffected enamel (Martinovic et al., 2015).

1.1.7. MIH - challenges in patient treatment

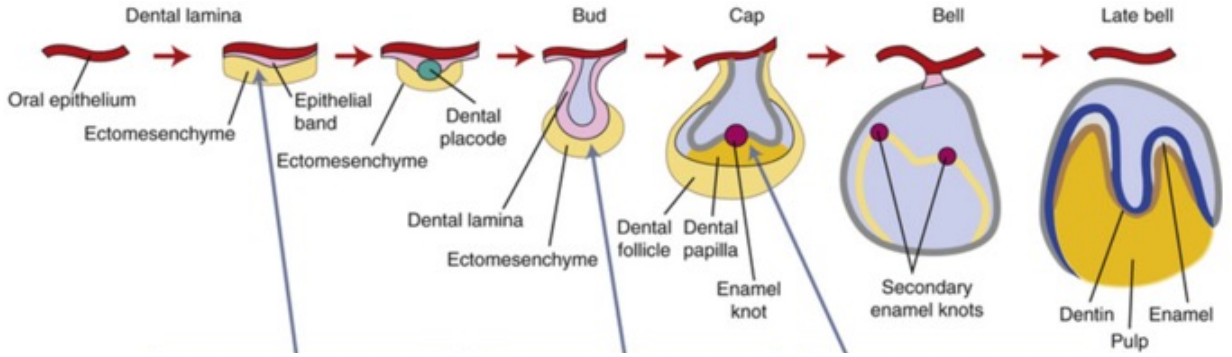
The treatment and management of MIH is challenging for the patient as well as the clinician. Children with severe MIH undergo dental treatment nearly ten times as often as children without MIH (Jalevik and Klingberg, 2002). Difficulties in achieving adequate anesthesia, the reduced mechanical properties of affected enamel, and a high risk for PEB are challenges to the success of interventions. Almost half of 18-year-olds with restorative treatment on FPMs due to MIH have restorations with unacceptable quality (Mejare et al., 2005). The economic aspects of treatment for MIH must be considered for the patients, their parents and public dental health services (Hubbard et al., 2017).

It is well documented that treatments for MIH-affected teeth may cause discomfort and pain (Rodd et al., 2007) and that painful treatments may cause dental fear and anxiety (DFA) and dental behavior management problems (BMPs) (Jalevik and Klingberg, 2002). Both DFA and BMP have been shown to be more common in children repeatedly treated for MIH (Jalevik and Klingberg, 2002). A longitudinal study of children treated for MIH, examined as adults, showed that these adults had undergone over four times more dental treatments and had significantly more common BMPs than the control group (Jalevik and Klingberg, 2012). However, the DFA level in these adults did not differ from that in the control group (Jalevik and Klingberg, 2012).

1.2. Development of enamel

1.2.1. Timing of tooth development in humans

The crowns of permanent teeth are formed between week 32 in utero up to the age of 14 years. In the primary dentition, the crowns mainly form prenatally. The formation of teeth starts at the cusp and ends at the cervical part (Proffit et al., 2018). Due to the tightly regulated process of tooth development and the absence of a capacity to repair defective enamel, clinicians can roughly estimate when an enamel disturbance occurred. The neonatal line is a band of incremental growth lines seen in histological sections of FPMs due to physiological changes at birth (Nanci,



Figur 5: Stages of tooth crown development (Nanci, 2017).

2017). This line is a solid reference to estimate the timing of insults, such as nutritional deficiency, diseases and their treatments, in tooth development.

The development of the tooth crown is, based on histological observations, commonly divided into five stages. The earliest histological indication of tooth development is the initiation stage, followed by the bud, cap, bell and late bell stages (Figure 5). In the permanent dentition, the initiation stage for FPMs starts before birth, and the development of the crown is finished 3-4 years later (Table 2). Closely related but occurring slightly later than the initiation stage for FPMs is the beginning of the development of incisors and canines. The second deciduous molar, affected in patients with HSPM, is the last tooth in the primary dentition to develop and is chronologically related to the development of FPMs (Table 2). The development of the permanent premolars and second and third molars initiates later. Regarding the development of MIH, it is assumed that the late bell stage is crucial. This stage includes the maturation and calcification of enamel (Nanci, 2017).

	Calcification begins		Crown completed		Eruption	
Tooth	maxillary	mandibular	maxillary	mandibular	maxillary	mandibular
Central	3 months	3 months	4½ years	3½ years	7¼ years	6¼ years
Lateral	11 months	3 months	5½ years	4 years	8¼ years	7½ years
Canine	4 months	4 months	6 years	5¾ years	11½ years	10½ years
FPM	32 weeks in utero	32 weeks in utero	4¼ years	3¾ years	6¼ years	6 years
Second deciduous molar	18 weeks in utero		11 months			

Table 2: Chronology of tooth development for permanent incisors, canines, FPMs and second deciduous molars (Schroeder, 1987, Proffit et al., 2018)

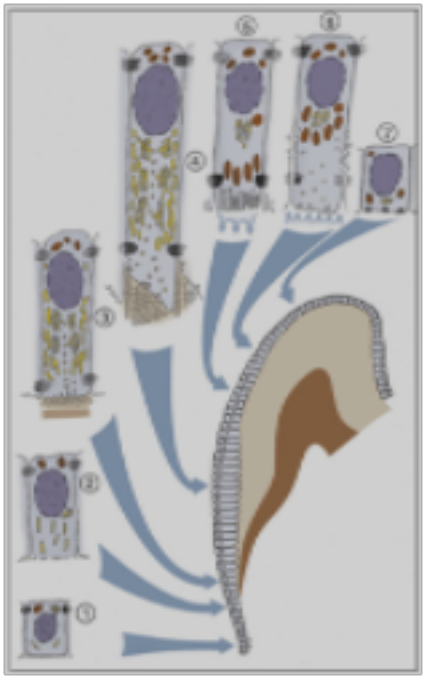
1.2.2. *The physiological formation of enamel*

The formation of enamel, also termed amelogenesis, is described in detail (Nanci, 2017). To understand the pathophysiological processes within amelogenesis, such as the characteristic hypomineralization of MIH-affected teeth, it is important to have a knowledge of the physiological processes.

Amelogenesis is a complicated process, taking from start to finish approximately thousand days for a permanent tooth. Disturbances during amelogenesis can result in qualitative and quantitative effects on enamel, depending on their timing and nature. Developmental insults of enamel are critical since enamel, unlike bone, is acellular and once formed is not remade (Lacruz et al., 2017).

Amelogenesis occurs in different stages (Reith, 1970, Nanci, 2017). In the first step, representing the presecretory and secretory stage, the enamel space is formed and partially mineralized up to 30%. In the second step, the maturation stage, the organic matrix and water are removed, and minerals are added (Nanci, 2017).

Each ameloblast exhibits a unique life cycle during amelogenesis, which has been described in seven different phases characterized by phenotypic changes that reflect the primary activity of the ameloblast at various times of enamel formation (Nanci, 2017). The first phase is the morphogenetic phase, followed by the histodifferentiation, initial secretory, and secretory phases, and finally maturation during the ruffle-ended and smooth-ended ameloblast phases. All these phases coexist since the onset of amelogenesis occurs first at the tip of the cusp and proceeds downward from the crown, finally initiating cervical amelogenesis (Figure 6). After amelogenesis is completed, the ameloblast remains in the so-called protective phase until the tooth erupts. In this last phase, the composition of enamel can still be modified (Nanci, 2017).



Enamel disturbances are usually detected years after amelogenesis is completed and the tooth is erupted. The effect of an insult on amelogenesis depends on its timing, duration and intensity. A disturbance within the secretory stage of amelogenesis will lead to hypoplasia, while hypomineralization will most likely emerge as a result of a disturbance during the maturation stage (Suckling, 1980).

Figur 6: Schematic representation of the six phases of an ameloblast: morphogenetic phase (1), histodifferentiation phase (2), initial secretory phase (no Tomes' process) (3), secretory phase (Tomes' process) (4), ruffle-ended (5) and smooth-ended (6) ameloblast phase of the maturation stage and finally the protective (7) phase (Nanci, 2017).

1.2.3. Pathophysiological aspects within amelogenesis

Anthropological studies have shown that dental developmental disorders were present at high frequencies in previous centuries. These disorders were predominantly linear enamel hypoplasias (30%) in addition to other enamel disorders (20%), such as diffuse opacities and Turner's teeth. Only 3% of dental development disorders were classified as MIH (Kuhnisch et al., 2016). The causes behind these disorders are strikingly different from those currently. The reasons are proposed to be malnutrition, severe illnesses with a lack of effective treatment opportunities and physical stress during childhood (Kuhnisch et al., 2016). In modern times, enamel defects are still common, affecting between 20 and 80% of the world's population (Lacruz et al., 2017). Linear enamel hypoplasias are present in clearly lower frequencies (4%), while dental developmental disorders such as dental fluorosis and MIH are quite common (Jalevik et al., 2018).

1.2.4. Pathological and medical conditions affecting amelogenesis

Factors that may cause enamel defects in the permanent dentition can be classified into local or systemic factors. Common local factors are traumatic injuries to the primary dentition as well as periapical osteitis of primary teeth (Pindborg, 1982).

Several systemic factors may affect amelogenesis. Examples are bacterial and viral infections; deficiencies in oxygen saturation related to delivery; treatment with antibiotics, chemotherapeutics or other medications; radiation exposure; low birth weight; prematurity; metabolic disturbances; and environmental pollution (Pindborg, 1982, Tredwin et al., 2005).

Common bacterial or viral infections that may affect amelogenesis are infections of the urinary tract, otitis media and upper respiratory infections. Amelogenesis can be directly affected by a bacteria/virus, or it can be indirectly affected by a high fever resulting from an infection (Beentjes et al., 2002, Kusku et al., 2008). In cases of respiratory illnesses, it is speculated that ameloblasts are affected by the lack of oxygen, which can cause enamel hypomineralization (van Amerongen and Kreulen, 1995). Viral infections such as mumps, measles and chicken pox have also been associated with enamel defects (Ford et al., 2009, Sonmez et al., 2013).

In addition, the treatment of an infection using, for example, antibiotics may affect amelogenesis. Regarding the antibiotic tetracycline, its ability to chelate calcium ions is well documented. The chelated calcium ions are incorporated within enamel mineralization and are clinically visible typically as discolorations of the teeth (Sánchez et al., 2004).

Metabolic disturbances, primarily those that affect mineral balance, may have an impact on amelogenesis. Examples of such metabolic disturbances are vitamin D deficiency, hypocalcemia, and coeliac disease (Souto-Souza et al., 2018, Uwitonze et al., 2020).

Several environmental pollutants are linked to defects in enamel development. Examples are high levels of fluoride, dioxins, or polychlorinated biphenyls such as bisphenol A (BPA) (Jedeon

et al., 2013, Alaluusua, 2010).

1.2.5. Inherited defects affecting amelogenesis

Amelogenesis is a complex process that is tightly controlled at the molecular level and involves thousands of genes (Wright et al., 2015). A disruption in the formation of enamel due to genetic factors is traditionally referred to as amelogenesis imperfecta (AI). AI is a genetically and phenotypically heterogeneous group of conditions that is characterized by the failure of normal amelogenesis. AI is seen in both the primary and permanent dentitions and will affect all teeth in the dentition.

In addition to AI is a group of hereditary, dermatological conditions associated with enamel defects. This group includes ectodermal dysplasia, epidermolysis bullosa and tuberous sclerosis complex (Freiman et al., 2009). The genetic background for these conditions and their impact on enamel development varies, although they all involve ectodermal tissues. It is speculated that genetic susceptibility is also associated with MIH (Jeremias et al., 2013b).

1.3. Etiology of MIH

1.3.1. Amelogenesis - etiology of MIH

It is widely accepted that MIH is associated with pre-, peri- and postnatal etiological factors (Lygidakis et al., 2008b, Alaluusua, 2010). Many different factors are suspected to cause MIH, but the exact pathogenesis is still unclear. Interestingly, all these factors seem to affect mainly FPMs but additionally incisors and canines, while premolars and second and third molars are not affected. This certainly depends on the timing of the insult taking place within the time span for amelogenesis of every affected tooth and obviously at a certain age of the patient.

The low number of MIH-affected dentitions shown in archaeological case series may support the hypothesis that MIH can be linked to contemporary living conditions and/or environmental factors. Examples are systemically administered pharmacological agents such as antibiotics and environmental pollutants such as polychlorinated biphenyls and dioxins (Kuhnisch et al., 2016). The most putative factors associated with MIH involve childhood illnesses, the use of medications during amelogenesis and environmental toxins. A multifactorial pathology is expected, and an individual hereditary susceptibility to MIH is suggested as well (Jeremias et al., 2013b).

1.3.2. Amelogenesis genes and susceptibility to developing MIH

Genetic susceptibility may be associated with MIH (Jeremias et al., 2013b). It is speculated that a genetically changed structure of matrix proteins leads to poor clearance by ameloblasts during calcification. A protein in focus within MIH genetic research is Kallikrein-related peptidase 4 (KLK4), which seems to have an important role during enamel maturation. Other elements within amelogenesis that could be affected genetically and are discussed in the literature are ion

transport (for example, Ca^{2+} transport), pH balance and the apoptosis of ameloblasts.

Genetic research, including family-based genetic association studies, has shown variations in proteins related to amelogenesis that are associated with a higher susceptibility to MIH development (Jeremias et al., 2013b, Kuhnisch et al., 2014c, Pang et al., 2020, Jeremias et al., 2016). Vieira (2019a) suggested that MIH could be explained by a gene-environmental model where multiple genes individually have minor effects on MIH but should be considered a causative factor in combination with environmental risk factors. Other authors suggested an interaction between amelogenesis and immune-related genes. An interaction of asthma and respiratory diseases has been evaluated, and the authors concluded that immune-related genes may have an additive effect on susceptibility to MIH (Bussaneli et al., 2019).

1.3.3. MIH - pre- and perinatal factors

Several pre- and perinatal factors are suspected to have an association with MIH. Infants born preterm, with low birth weight or cesarean delivery, associated with insufficient oxygen levels, seem to have an increased risk of MIH (Brogardh-Roth et al., 2011, Silva et al., 2016, Souza et al., 2012, Jacobsen et al., 2014, Aine et al., 2000). Additionally, maternal smoking, stress and mothers' use of medications such as antibiotics, cortisone and chemotherapy during pregnancy have been mentioned as possible causative factors (Whatling and Fearne, 2008, Silva et al., 2016, Fagrell et al., 2011). Due to the heterogeneity of these factors, as well as the close connection between many of them, it is challenging to point out any specific pre- or perinatal determinants of MIH.

Children born preterm are significantly more often affected by MIH or HSPM than children born at term. The exact mechanisms underlying these enamel defects are not fully understood (Aine et al., 2000, Brogardh-Roth et al., 2011, Lima et al., 2020). However, it is hypothesized that a lack of bone minerals such as calcium and phosphorus or a lack of vitamin D could be etiological factors behind dental developmental defects of the primary and permanent dentitions in preterm children (Aine et al., 2000, Kuhnisch et al., 2015, van der Tas et al., 2018, Seow, 1996).

The fetus normally accumulates a sufficient amount of calcium during the last trimester of pregnancy. During the first days of life, newborns experience neonatal hypocalcemia. Full-term infants show only slightly decreased calcium concentrations, which usually return to normal within 5-10 days (Salle et al., 2000). However, preterm infants do not have enough accumulated calcium (Alaluusua, 2010, Lygidakis et al., 2008b), and breast milk does not contain enough calcium and phosphorus to ensure optimal mineral retention in the bone tissue (Koo and Tsang, 1991, Schanler and Abrams, 1995). It is suspected that inadequate mineral retention leads to disturbances in amelogenesis. It seems that the lower the gestational age is at birth, and the longer the duration of parenteral nutrition is, the higher the number of affected teeth will be. Mineral supplementation for children born preterm may diminish, but not totally prevent, enamel defects (Aine et al., 2000).

Vitamin D deficiency is common in low-birth-weight, preterm infants (<1500 g) (Munshi

et al., 2018, Burris et al., 2014). Vitamin D binds to the vitamin D receptor (VDR), the most highly expressed steroid receptor in the enamel maturation phase, and both vitamin D and VDR are closely associated with enamel mineralization (Babajko et al., 2017). A balanced concentration of vitamin D during odontogenesis is important to avoid disturbances in enamel maturation (Dhamo et al., 2019). However, the results of studies on whether a lack of perinatal vitamin D status is associated with MIH and/or HSPM show conflicting results (Kuhnisch et al., 2015, van der Tas et al., 2018). Vitamin D, as well as mineral supplementation, is recommended, especially for infants born prematurely. Further studies are needed to clarify the effects, as well as the optimal doses, of vitamin D supplementation to avoid subsequent enamel defects due to a possible vitamin D deficiency (Aine et al., 2000).

1.3.4. MIH - postnatal factors

Several postnatal factors have been proposed to be associated with MIH (Alaluusua, 2010, Serna et al., 2016, Silva et al., 2016, Tourino et al., 2016). Health problems or medical treatments within the first year of life seem to have a higher statistically demonstrated influence on the prevalence of MIH than health problems during the second and third years of life (Jalevik et al., 2001b).

An association between long-term breastfeeding and MIH seems to exist (Alaluusua et al., 1996a). Environmental contaminants such as dioxin or polychlorinated biphenyls (PCBs) are lipophilic substances that can be passed to the newborn via mother's breast milk. It seems reasonable to suggest that a dose-response relationship exists between perinatal PCB exposure and enamel defects (Wang et al., 2003). Even if the pollution of these environmental contaminants has decreased during the past decades in Europe, exposure to toxins through breastfeeding might be considered a potential factor for MIH, especially in regions with high pollution (Alaluusua et al., 1996b, Kuhnisch et al., 2014b).

Early respiratory diseases such as asthma, pneumonia and upper respiratory infections, as well as otitis media, may have an impact on the risk of developing MIH (Jalevik et al., 2001b, Kuhnisch et al., 2014b, Flexeder et al., 2020). It is difficult to discriminate whether the infection itself or the systemic treatment of the infection has a greater influence. Frequent use of aerosol therapy with, for example, corticosteroids in early childhood is a risk factor for the development of MIH (Loli et al., 2015). Animal research has shown that corticosteroids can disrupt amelogenesis (Pawlicki et al., 1992). Antibiotics, which may potentially cause MIH, are widely used within the treatment of respiratory diseases and other bacterial infections (Laisi et al., 2009, Kuhnisch et al., 2014b).

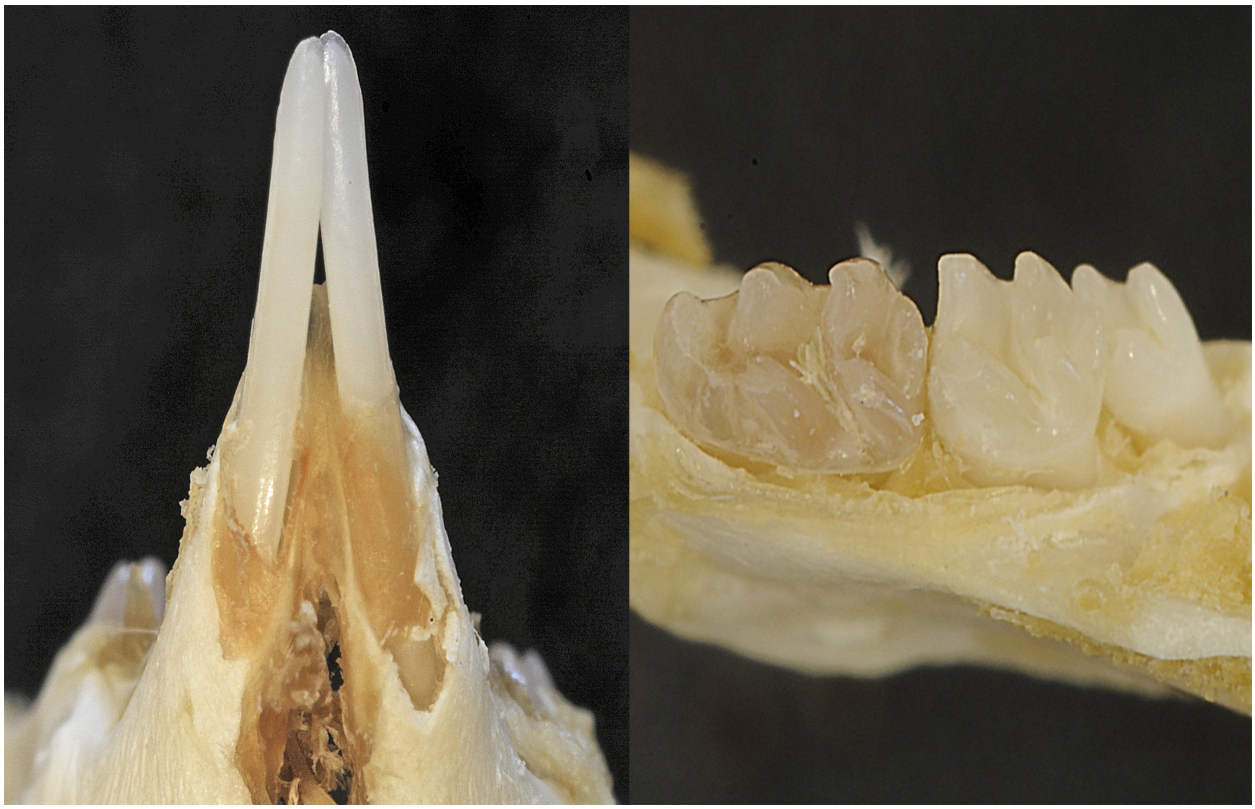
Drugs used in chemotherapy for the treatment of epilepsy and the use of antibiotics prescribed during the first years life are suspected to be associated with MIH (Serna et al., 2016). In addition to vitamin D deficiency (Kuhnisch et al., 2015), vitamin A deficiency is proposed to be

a triggering factor for MIH (Mishra and Pandey, 2016).

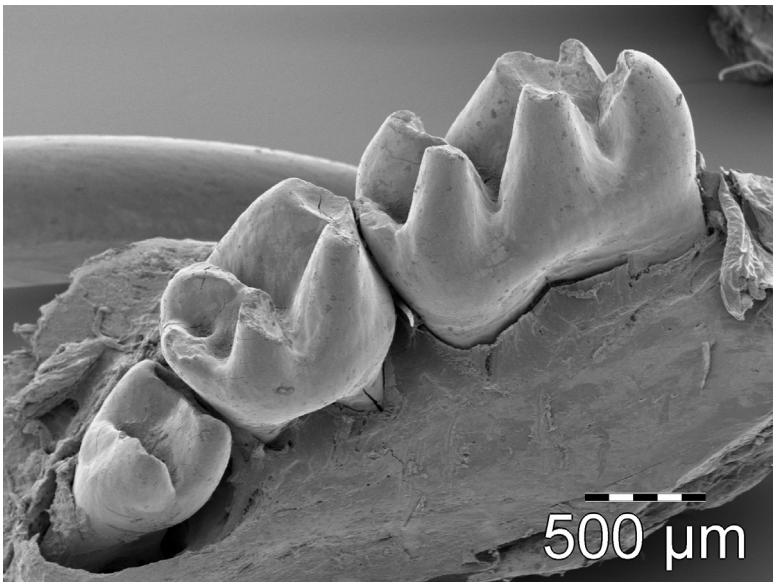
1.4. The role of animal studies in enamel research

A considerable proportion of contemporary knowledge about amelogenesis and its pathogenesis is based on animal research (Fejerskov, 1979). Animal models, both in vitro and in vivo, are essential to obtain an understanding of the basic biology of amelogenesis as well as to evaluate the effects of various medications and toxins on amelogenesis. Important requirements are that tooth development should be comparable with that of humans, that tooth development should take a reasonable time and that nutritional requirements should be well known (Fejerskov, 1979).

Rodent incisors are especially evaluated since they erupt continuously during the lifespan of the animal, allowing us to study all stages of amelogenesis within the same tissue section. Profound knowledge about the amelogenesis time schedule as well as the most important physiological processes could be gained by animal research, mainly performed on rodents. The enamel basic structure, as well as the mode of formation, is comparable with the enamel structure of humans (Warshawsky et al., 1981, Moinichen et al., 1996). Rodent molars are quite comparable to human molars regarding shape and morphology (Figure 7, 8 and 9) (Tucker and Sharpe, 2004). In contrast to the molars, the morphology of rodent incisors differs obviously from that of human incisors (Figure 9c&d). Only the buccal surface of a rodent incisor is covered with enamel, and the enamel on this surface is continuously formed throughout a rodent's lifetime. The eruption rate is 200 μm/



Figur 7: Macrophotography of lower incisors (left) and lower first, second and third molars (right).



Figur 8: Scanning electron microscopy of an 18-day-old mouse from the study group: a left mouse mandible, lingual view with the first mandibular molar on the right side.

day for mandibular and 160 µm/day for maxillary incisors (Sidaly et al., 2015b).

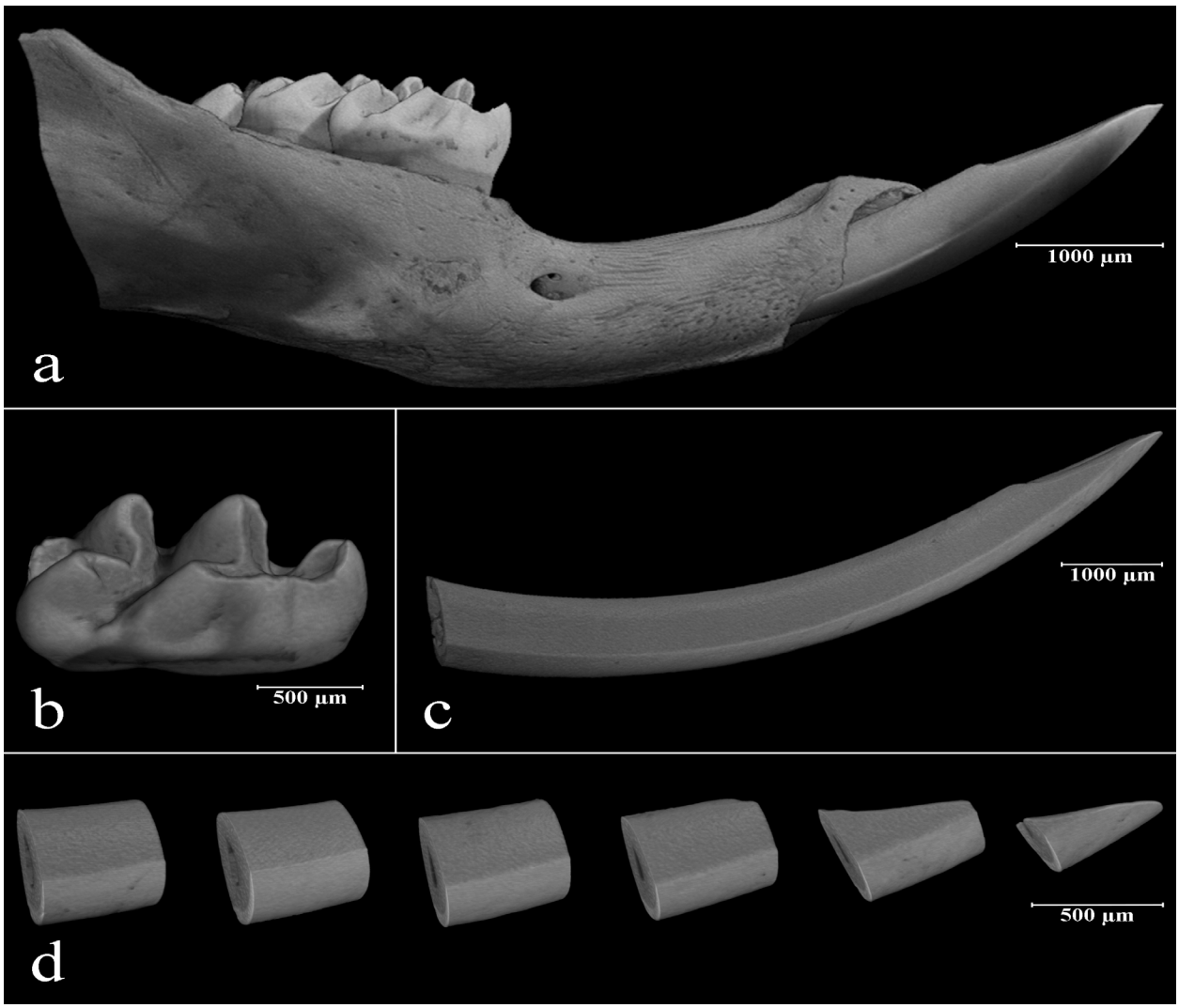
The development of transgenic animal models allows the study of the function of enamel matrix proteins during enamel formation. An important benefit of animal models in general is the possibility of standardizing them for valid comparative studies. However, the transferability to findings to humans must be evaluated critically, and the interpretation of results based on animal research should always be taken with caution.

1.4.1. MIH - in vitro and in vivo experiments using animal models in enamel research

Many different study designs have involved rodents within enamel research. One approach is in vitro studies using tooth germs from embryos that are cultured for several days under exposure to different agents. The developmental stage of mouse first molars at embryonic day 18 (E18) corresponds approximately to that of FPMs in humans slightly before birth (Sahlberg et al., 2013). The advantage of this study approach is the isolated examination of one or several factors affecting amelogenesis. This approach has been used to examine the effect of the antibiotic amoxicillin and fluorides, alone or in combination, on enamel formation (Sahlberg et al., 2013, Laisi et al., 2009). The results using this approach should be interpreted with care because the in vitro environment differs from the natural environment.

In vivo studies using rodents such as mice and rats, both pups and adult animals, are common within enamel research. Pregnant animals can be used if prenatal intervention is required. Many results due to physiological as well as pathophysiological processes within amelogenesis have been gained using incisors since these teeth show all stages of amelogenesis at the same time.

Several results within MIH research using animals in experimental models have been



Figur 9: Three-dimensional reconstruction of slices from micro-CT of 18-day-old mice from the study group: right mouse mandible, buccal view (a); isolated crown of the first mandibular molar (b); isolated crown of a mandibular incisor (c); a mandibular incisor divided into six segments (S1–S6) (d).

published. Factors such as fever; environmental toxins such as dioxin and PCBs; antibiotics such as amoxicillin, erythromycin, and macrolide; hypoxia; and hypocalcemia seem to affect amelogenesis and may be related to MIH (Elfrink et al., 2014, Alaluusua, 2010). Amelogenesis seems to be sensitive to these factors only during a specific time window, which corresponds to the appearance of MIH in humans. This fact has been demonstrated in rats treated with BPA starting with perinatal exposure until day 30 of life (Jedeon et al., 2013). The rats in this study group showed enamel hypomineralization, similar to human MIH. Another study group was exposed to BPA starting with perinatal exposure until day 100 of life. The erupting enamel does not show more enamel hypomineralization in comparison to the study group with BPA exposure until day 30 of life. The authors suggest that BPA disrupts normal protein removal only during a specific time window. The dependence on the timing of an insult to result in enamel hypomineralization is

characteristic of MIH, but the causal link for this fact is not yet fully understood.

Animal experiments could be used to improve the knowledge about the pathophysiology behind MIH. The validation of an animal model in MIH research does not seem to exist.

1.5. Overall aim of this thesis

The general objectives of this thesis were as follows:

A. To investigate the MIH prevalence, the distribution of affected teeth and MIH severity in 16-year-olds from northern Norway.

B. To test a possible association between hypoxia during birth and MIH in a group of 8- to 10-year-olds.

C. To determine whether the use of high-dose ampicillin and gentamicin in combination, usually given to preterm infants with suspected sepsis, could cause mineralization disturbances in dental enamel using a newborn mouse model.

2. Materials and Methods

2.1. Paper I

2.1.1. Study population

This study regarding the prevalence of MIH in northern Norway was part of a larger epidemiological general health project called "Fit Futures" conducted in Troms country (Jacobsen et al., 2012, Winther et al., 2014). In September 2010 to May 2011, all first-year upper-secondary-school students from the two neighboring municipalities of Tromsø (7 schools) and Balsfjord (1 school) were invited to join this cross-sectional health survey.

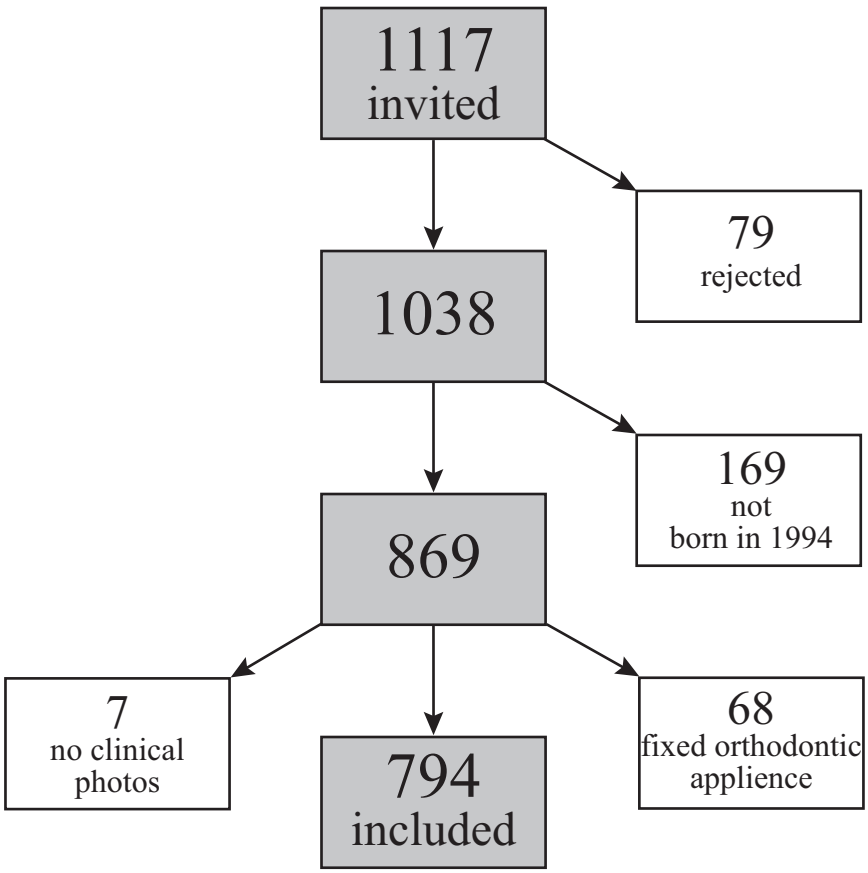
The Norwegian Data Protection Authority and the Regional Committee of Medical and Health Research Ethics (reference numbers 2009/1282 and 2011/1702/REK, respectively) approved the study in July 2010 and October 2011, respectively.

Recruitment took place at schools, and information was presented orally, electronically and by distributing a brochure to students and parents/guardians. Students interested in participating in the study confirmed this interest via an internet link sent to their personal e-mail address and signed a written consent upon arrival for the examination. All participants who had turned 16 years of age by the day of examination as well as the guardians of the younger participants gave written informed consent. To obtain a high participation rate, the survey was conducted during school hours. The participants were transported from the schools to the examination stations at the university by mini-buses, and a 200 NOK (US $35) voucher was handed out.

The invited cohort included 1,117 participants, mainly between 15 and 19 years old, and a total of 1,038 adolescents (508 girls and 530 boys) accepted and participated (Figure 10). Thus, the Fit Futures attendance rate was 92.9% (Winther et al., 2014).

The oral health part of Fit Futures included all individuals born in 1994 and was performed at the University Dental Clinic of the Arctic University of Norway (UiT) in Tromsø (Jacobsen et al., 2016). A clinical examination with two bitewing radiographs, replacing the annual dental examination at the Public Dental Health Service (PDHS), was carried out by an experienced and calibrated dentist assisted by a dental nurse. The participants were examined in a dental chair. Dental lighting, mirrors and blunt probes were used to examine all visible tooth surfaces. Additionally, impressions of both jaws were taken.

As a part of the clinical examination, eight photographs (Canon EOS 60D; Canon 105 mm; Sigma EM-140 DG) were taken by experienced trained dental nurses or dentists in the following order: the buccal surfaces of the teeth in the first and fourth quadrants (#1), the corresponding surfaces in the second and third quadrants, the buccal surfaces of maxillary and mandibular anterior teeth (#3), the occlusal surfaces of the upper teeth (#4 & 5) and lower teeth (#6 & 7) and the palatal surfaces of the upper anterior teeth (#8) (Figure 11). All photographs were taken with the use of



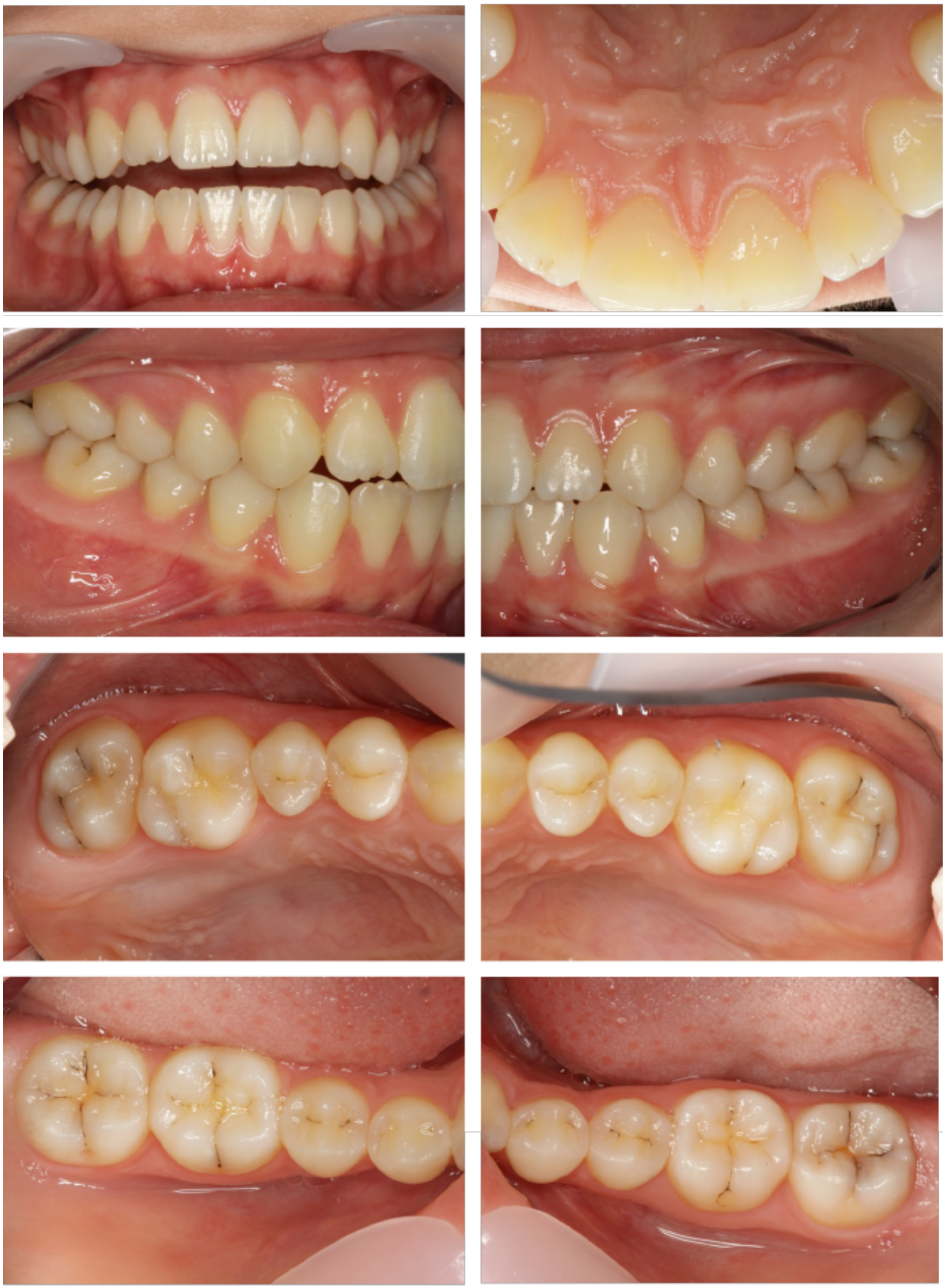
Figur 10: Participant inclusion flowchart for Fit Futures.

cheek retractors. Additionally, for picture #3, an occlusal mirror was used, and for photographs #5 to #8, a buccal/lingual double-ended photography mirror was used. To enable the evaluation of the complete buccal surfaces of incisors, picture #1 was taken with slight mouth opening. The tooth surfaces were kept moist, avoiding abundant saliva. All photographs were critically evaluated and repeated if required.

In the present study, the data from the 869 individuals born in 1994 were evaluated. In total, 7 participants were excluded due to missing or inadequate clinical photographs, and 68 participants were excluded due to fixed orthodontic appliances in one or both jaws. In total, 794 individuals (380 girls and 414 boys) with complete datasets were included in the present study (Figure 10).

2.1.2. Diagnostics of MIH

The clinical photographs of the 794 adolescents were shown on a flat screen in a room with indirect, standardized lighting and examined independently by three experienced and calibrated dentists. In line with the EAPD guidelines for MIH (Lygidakis et al., 2010), the buccal, occlusal and palatal/lingual surfaces of all FPMs were examined, as well as the labial surfaces of all central and lateral incisors and canines. Characteristics such as opacities (white/cream/yellow-brown color), PEB, atypical restorations and extractions judged as being due to MIH were recorded. Opacities >1 mm were registered (Lygidakis et al., 2010). The examiners recorded the data individually and



Figur 11: Example of the clinical photographs of one participant.

independently. A joint score was decided for each recording, and a consensus was reached through discussion when individual scores differed. The classification of MIH-affected teeth and surfaces was based on the most severe diagnosis recorded (opacity\PEB\atypical restoration).

Affected MIH teeth were given a dichotomous score classifying the lesions as mild or severe. Surfaces or teeth with opacities only were defined as mild (grade 1). Surfaces or teeth with PEB, atypical fillings and teeth that had been extracted were defined as severe (grade 2). If an opacity and PEB or restoration occurred on the same surface, it was scored as severe (Lygidakis et al., 2010).

To calculate the intra- and interexaminer agreement, a re-examination was performed one month later. The examiners repeated the registrations of 10% (n = 11) of photographs randomly selected with MIH diagnoses and 10% (n = 68) without MIH diagnoses. The photographs were mixed and "blinded" before the re-examination. The codes that were used in the kappa calculations (tooth level) were 0 = MIH free and 1 = MIH affected.

2.1.3. Statistical analyses

The data were analyzed using the SPSS package version 21.0 (IBM SPSS Inc., Chicago, IL, USA). Interobserver analyses (kappa statistics) were performed with MedCalc version 13 (MedCalc Software, Ostend, Belgium).

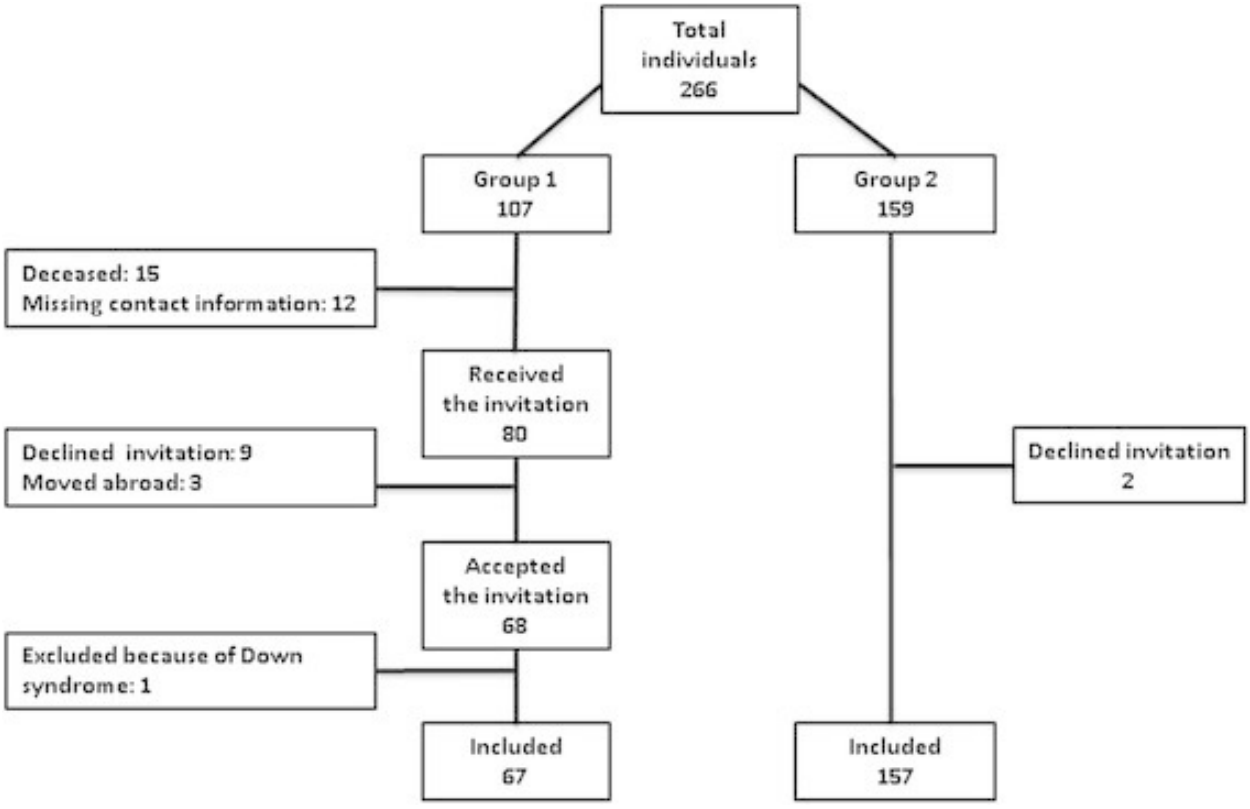
2.2. *Paper II*

2.2.1. *Sample population*

This cross-sectional, case-control study was conducted in Oslo and Akershus counties, southern Norway.

The Norwegian Data Protection Authority and the Regional Committee of Medical and Health Research Ethics (reference number 2013/1072/REK sør-øst) approved the study. Informed consent was signed by each child's parents prior to data collection.

Two groups of children were investigated based on clinical and photographic examinations of 8–10-year-old children (n = 266) (Figure 13). The study group had been diagnosed with birth asphyxia, defined as an Apgar score ≤ 5 (Group 1) 5 min after delivery. The Apgar score is a vitality index, ranging from 0 to 10, that summates five clinical signs, heart rate, respiratory effort, reflex irritability, muscle tone and color, each given a score of 0, 1, or 2. Today, the Apgar score is routinely determined at Oslo University Hospital for every newborn at one, five and ten minutes after birth. An Apgar score of 7-10 is considered normal, while a lower score is an indicator of decreased vitality (Apgar, 2015). The study group comprised all children born in 2004-2006 and admitted to the Neonatal Intensive Care, Ullevål, Oslo University Hospital, with an Apgar score ≤ 5 at 5 min. Originally, this group comprised 107 children, of whom 80 were invited to participate and 67 were included in the final study group (Figure 12). The national population



Figur 12: Flowchart of individuals enrolled in the study. Group 1 represents the study group and group 2 the control group.

register (Folkeregisteret) was contacted to confirm the contact details obtained from the medical records. The control group (Group 2) comprised 157 8- to 10-year-old randomly selected children healthy at birth attending the Public Dental Health Service (PDHS) in Lillestrøm in the county of Akershus (Figure 12). The controls were age-matched with the study cases (born in 2004–2006). The percentages of boys and girls in the study group were 53.0% and 47.0%, respectively, and the corresponding percentages in the control group were 49.7% and 50.3%. The mean age in the study group was 9.0 years (SD 0.84, range 8–10), and that in the control group was 9.0 years (SD 0.80, range 8–10).

2.2.2. Data collection and analysis

A structured medical history questionnaire was sent or handed to the parents of the participating children. The questionnaire included data on maternal health, pregnancy, delivery, prematurity, breastfeeding, the use of antibiotics and infections during early childhood, as described in previous studies (Koch et al., 1987, Laisi et al., 2009). The children were examined in a dental clinic for the presence and severity of MIH by two dentists (RS and AS). Dental lighting, mirrors and blunt probes were used. After the clinical examination, seven photographs (Canon EOS 60D; Canon 100 mm; Canon Ring Lite MR -14EX) were taken according to a standardized protocol in the following order: the buccal surfaces of the teeth in the first and fourth quadrants (#1), the corresponding surfaces in the second and third quadrants (#2), the buccal surfaces of the upper and lower anterior teeth (#3), and the occlusal surfaces of the upper teeth (#4 & 5) and lower teeth (#6 & 7). Most of the photographs were taken by the same photographer. To enable the evaluation of the complete buccal surfaces of incisors, picture #1 was taken with slight mouth opening. The tooth surfaces were kept moist, avoiding abundant saliva.

For the diagnosis of MIH based on intraoral photographs, the photographs were displayed on a Sony 55" TV monitor (Sony Model no. KDL 55WB02A LCD-TV, Sony Corporation, Japan) in a room with covered windows and dimmed room lighting. The intraoral photographs were then evaluated for enamel defects by three independent and blinded observers and authors (AS, ABS and IE).

In a few cases, when the quality of the photographs was not optimal due to poor patient compliance, the clinical diagnosis was taken into account.

Prior to the clinical examination as well as the diagnostics according to MIH based on the clinical photographs, a calibration exercise was conducted among all authors. The examiners applied the diagnostic criteria that were agreed upon at a workshop organized by the EAPD (Lygidakis et al., 2010). All second primary molars and index teeth, which included FPMs and all permanent incisors, were examined for demarcated opacity (white/cream/yellow-brown color), PEB, atypical restorations and extractions due to MIH. Opacities >1 mm in diameter were registered. The term MIH was used for dentitions with one or more hypomineralized FPMs with or

without hypomineralized permanent incisors (Weerheijm and Mejare, 2003). When more than one defect occurred on a tooth surface, the most severe defect was recorded. Sum scores of recordings on FPMs were calculated to express the severity of MIH in each individual. For each person with MIH, the sum score theoretically could range from 1 to 8, as each FPM received a score of 1 for opacity or 2 for PEB, an atypical filling or an extraction due to MIH. The final diagnosis of MIH was based on consensus among the observers based on the clinical photographs and in a few cases in combination with the clinical examination.

One month later, the clinical photographs of a subsample of children (n = 35, 15.5%) were re-examined in the same room under identical lighting conditions on the same TV monitor. The photographs were mixed and "blinded" before the re-examination. Cohen's kappa was calculated for inter- and intraexaminer agreement at the tooth level using the codes 0 = MIH free and 1 = MIH affected.

2.2.3. Statistical analyses

The data were analyzed using SPSS version 22.0 (IBM SPSS Inc., Chicago, IL, USA). The chi-square test was used for comparisons. Significance was set at $p < 0.05$. Interobserver analyses (kappa statistics) were performed with MedCalc version 13 (MedCalc Software, Ostend, Belgium).

2.3. Paper III

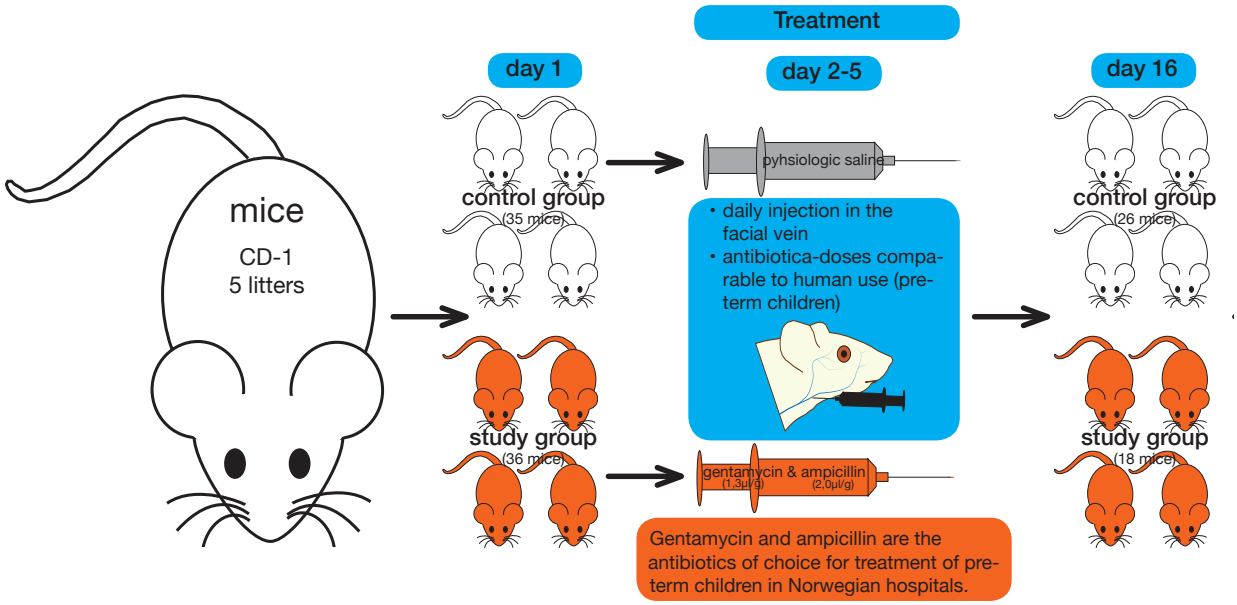
2.3.1. Animal model

To meet the requirements for reliable power analysis calculations, a spontaneous effect on amelogenesis within a control group must be covered. No data are available regarding spontaneous affection on amelogenesis in neonatal mice, so enamel affection in line with MIH were estimated to be 5% in mice without intervention. The effect on the study group was estimated to be 30%, which is in line with the effect on children born preterm and commonly treated with high-dose antibiotics (Brogardh-Roth et al., 2011). Based on a power analysis, the study was planned to be conducted on a total of 80 mice, with 40 mice each in the control and study groups. The project was started with two pregnant mice followed by another three shortly thereafter. Five pregnant mice (CD-1 strain) were maintained on a 12-h light-dark cycle at 21°C and at a relative humidity of 55%. The mice were given standard laboratory fodder and water ad libitum. The animals were kept according to the regulations of the Norwegian Gene Technology Act of 1994, and the experiment was approved by the Local Veterinary Service (FOTS ID 8325). This study conformed to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

In total, 71 neonatal mice were obtained and randomly allocated to a study group (n=36) and a control group (n=35) (Figure 13). Cutting the tail tip marked the mice of the control group. Starting at postnatal day one (P1) or two (P2), drugs (described later) were intravenously injected for four days in the morning as described by Glascock et al. (2011). Filtered green food dye (1:100) was added to the solutions to control access to the intravenous injection. The green dye rendered the drug distribution visible, as the normal pink skin color of the neonates was converted to green. Before injection, the facial vein was made visible by using a Wee Sight™ transilluminator (Philips, Amsterdam, Netherland). The solution was slowly injected with an insulin syringe (Micro-Fine™ 8 mm x 30 G; Becton Dickinson, New Jersey, USA) using a 2.5X magnifying visor loupe (Lactona, Bergen op Zoom, Netherland). Bleeding from the injection site was stopped by applying pressure with a gauze swab. After the transient stress from the injection was relieved, the mice were returned to the cage and kept under standard conditions.

All neonatal mice were sacrificed by cervical dislocation at the age of P16 to P18. The jaws, including incisor and molar teeth, were dissected out and fixed in 70% ethanol. After fixation, the jaws were thoroughly cleaned by gentle brushing in running tap water using a stereomicroscope with a light source (SteREO Discovery V8 & SteREO CL 1500 ECO; ZEISS, Oberkochen, Germany). Teeth with visible iatrogenic decay accidentally inflicted during preparation were excluded from the study.

All medicines were purchased from the hospital pharmacy (Sykehusapoteket Rikshospitalet, Oslo, Norway) and prepared for the study group as follows: the ampicillin solution was prepared

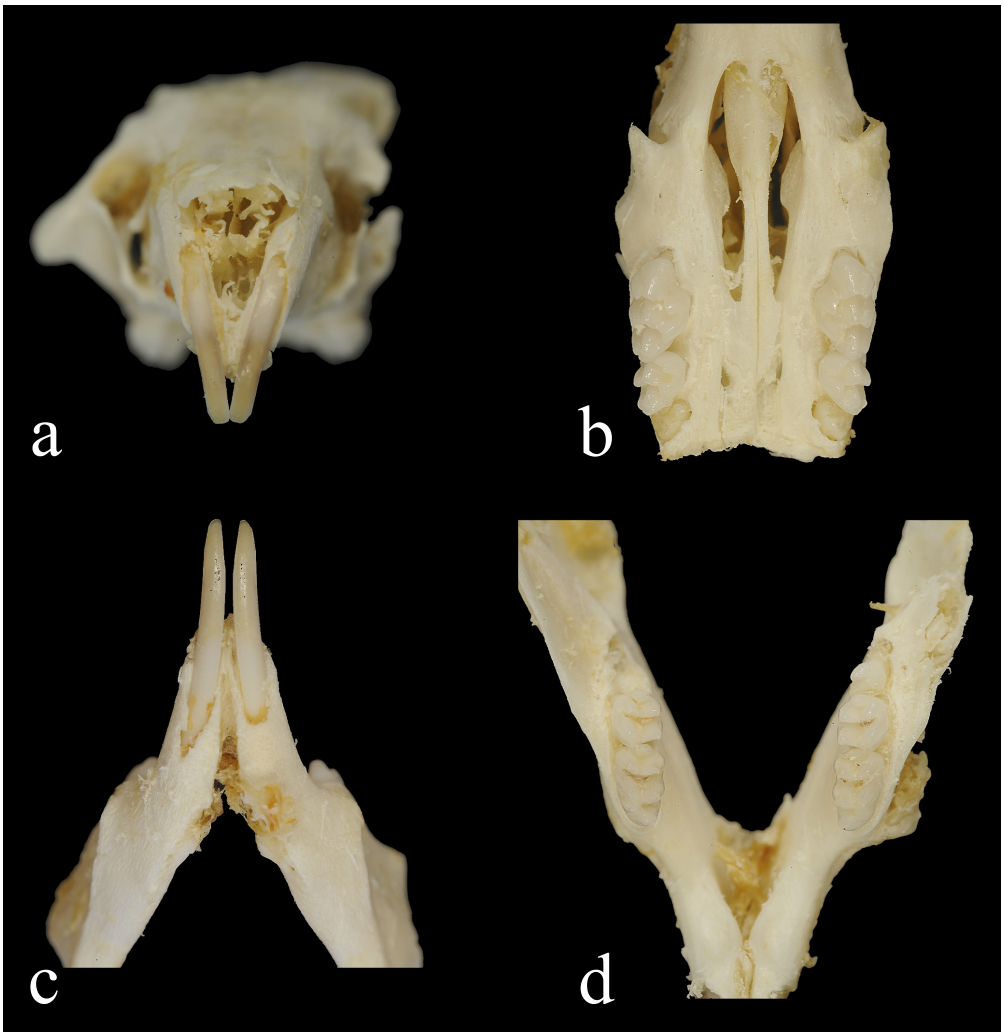


Figur 13: Experimental setup and treatment.

by mixing powder and sterile physiological saline according to the manufacturer's instructions (Pentrexyl; Bristol-Myers Squibb, Lysaker, Norway) and adding sterile filtered green food dye (1:100). Gentamicin (Gentamicin; B. Brown, Melsungen, Germany) was administered using a prefabricated solution (3 mg/ml) for injections. Before use, both antibiotic solutions were mixed according to the requested dose: ampicillin solution (200 mg/kg) 2.0 µl/g mouse body weight and gentamicin solution (4 mg/kg) 1.3 µl/g mouse body weight, equivalent to what is given to preterm infants in Norwegian hospitals. The injected volume per mouse was between 6.6 µl and 15.2 µl, corresponding to the body weight (2.0–4.6 g). For the control group, sterile saline was prepared with sterile filtered green food dye (1:100), and a volume corresponding to 3.3 µl/g mouse body weight was injected.

2.3.2. Macrophotography

As a part of the enamel examination, photographs were taken using a digital single-lens reflex camera with a macrolens (D700 & AF Micro Nikkor 60 mm, 1:1; Nikon, Tokyo, Japan) and extension tubes (DG 12, 20 and 36 mm; Kenko Tokina, Tokyo, Japan) with standardized camera settings (ISO 200, f32, 3 sec): the labial aspects of the upper and lower incisors and the occlusal surfaces of the first upper and lower molars were photographed (Figure 14). The photographs were projected on a flat screen in a room with indirect, standardized lighting and examined individually and independently by three experienced dentists. The photographs were randomized and blinded before the examination. Enamel disturbances such as demarcated opacities (white, yellow and brown color), hypoplasias and other enamel defects were recorded. A joint score was decided for each recording, and consensus was reached through discussion when individual examiners differed.



Figur 14: Photographs of teeth from mice in the study group: (a) maxillary mouse incisors; (b) maxillary mouse molars; (c) mandibular mouse incisors; (d) mandibular mouse molars.

2.3.3. Micro-CT imaging

A micro-CT scanner (SkyScan 1172; Bruker, Brussels, Belgium) was used to obtain three-dimensional images of mouse incisors and first molars to determine the enamel volume and density. The scanning protocol and parameters for human teeth described in previous studies (Johnsen et al., 2016) were used with adjustments to the requirements for the analysis of murine dentitions. Samples were mounted vertically in customized tubes. Hydroxyapatite phantoms with known density were used to calibrate the instruments. The scanning parameters were 4.96 μm isotropic pixel size with a medium camera resolution and X-ray source (100 kV, 100 mA, 10 W) using a 0.5 mm Al filter. Samples were rotated 360° around their vertical axes, with a 0.4-rotation step and frame averaging of three. A flat field correction was performed before every scan. The X-ray attenuation coefficients for serial coronally oriented tomograms were reconstructed with SkyScan software Nrecon (Bruker, Brussels, Belgium) using a modified algorithm, with a beam hardening

of 20%, a ring artifact correction of 12 and an attenuation coefficient range of 0.00 to 0.05. The grayscale was first defined using control mouse enamel, allowing the isolation of enamel alone without any other structure. The resulting histogram was used to determine a binary threshold of 60 to 255 for hard tissue, including enamel and dentine, and 130 to 255 for isolated enamel.

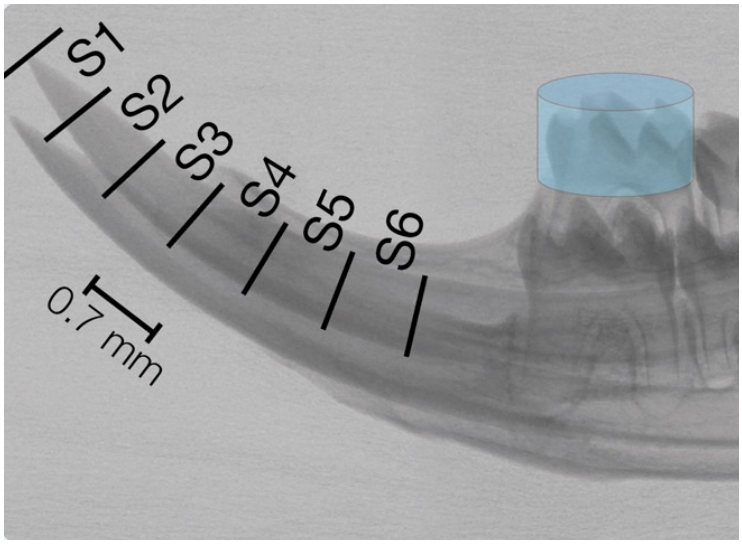
Before analyzing the enamel, first molars and incisors were isolated from the surrounding tissue prepared digitally using DataViewer (Bruker, Brussels, Belgium). The crown of the molars was isolated from the root at the cementoenamel junction in a standardized way (Figure 15). Incisors were divided into six segments, each being 0.7 mm long starting at the incisal edge with S1 and ending at the most apical part of the tooth representing S6 following a standard protocol (Figure 9). All images were inspected visually and with micro-CT, and iatrogenic artifacts were registered.

SkyScan software CTan and CTvol (Bruker, Brussels, Belgium) were used to determine enamel quality and quantity. The enamel was analyzed regarding mineral density and the proportion of the enamel's object volume (vol%) of the tooth's or segment's total hard dental tissue.

2.3.4. Statistical analysis

The results (enamel volume and density) are expressed as the mean ± SD of independent experiments in the upper and lower first molars and in every single segment (S1 to S6) of incisors. Shapiro-Wilk's test was used to test normal distribution. If normality failed, values were transformed using log10, √X or 1/X, and Shapiro-Wilk's test was repeated. One-way ANOVA was used to analyze the variance.

Welch's t-test was performed in cases where both normality and variance failed. Student's t-test was used in cases with normal distribution and equal variance. The Mann-Whitney U-test was performed when normality failed, and the variance was equal. Results were considered statistically different if $p<0.05$. All calculations were carried out using SPSS version 24.0 (IBM SPSS Inc., Chicago, USA).



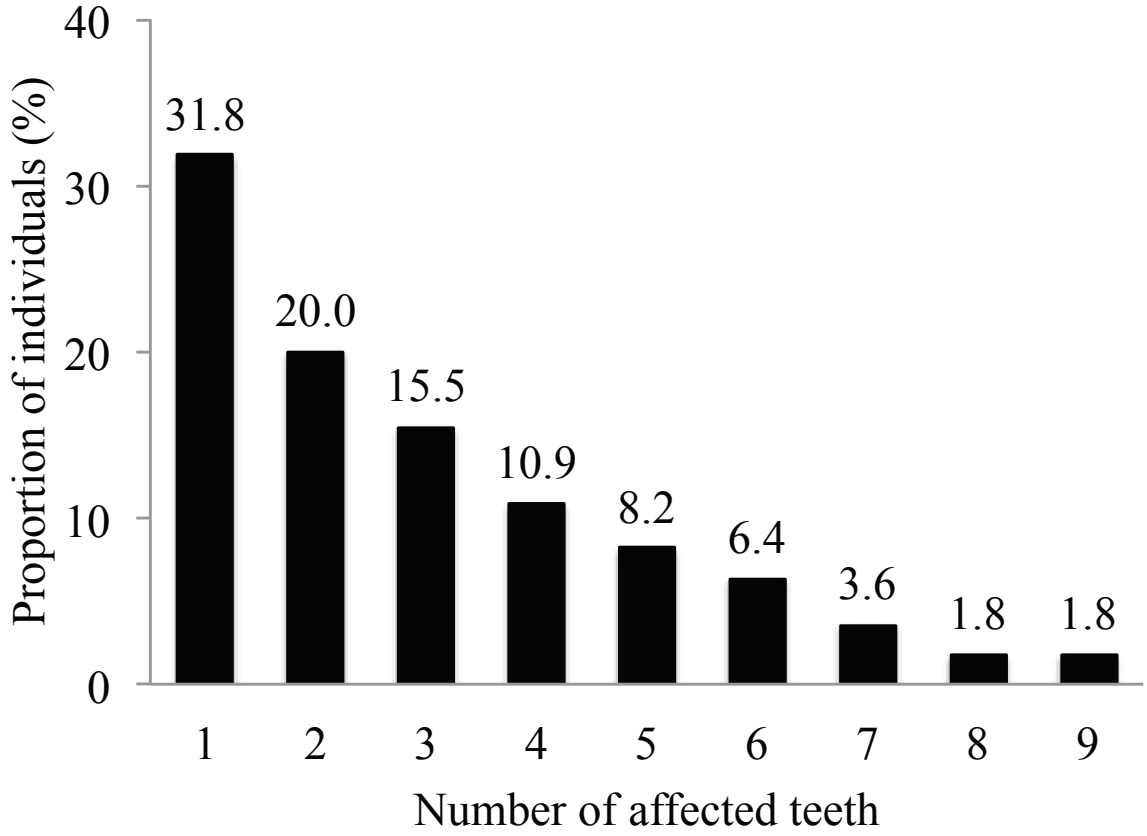
Figur 15: Illustration of the isolation of the crown (blue cylinder) and the six segments (S1-S6).

3. Summary of results

3.1. Paper I. MIH prevalence, the distribution of affected teeth and MIH severity study

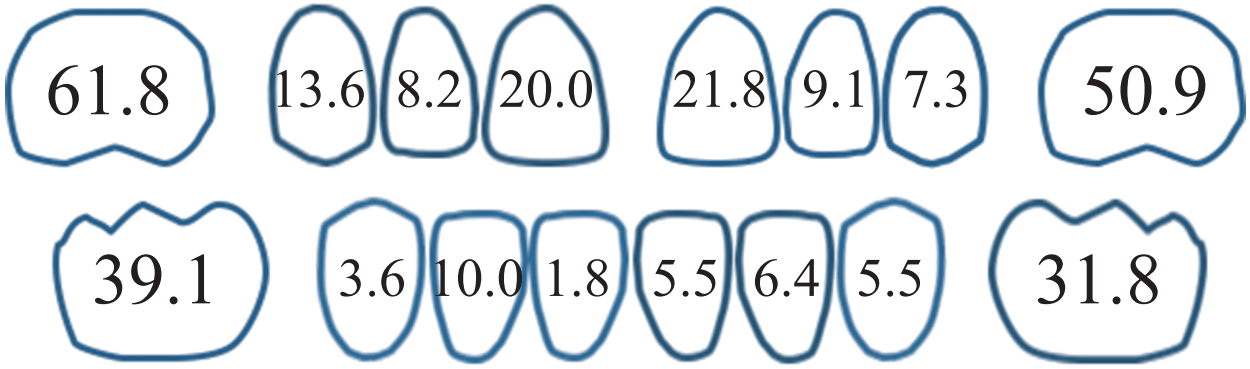
The mean age of the participants was 16.6 years (range 15.8–17.3 years). Interexaminer and intraexaminer agreement for MIH diagnosis at the patient level, measured by Cohen's kappa (95% CI), was in the range of 0.91–0.99 and 0.86–0.88, respectively.

The prevalence of MIH was 13.9% (110 of 794 participants). Girls were more often affected than boys (16.3% vs. 11.6%; P=0.054). The number of affected teeth among the individuals with MIH is presented in Figure 16. The mean numbers of affected index teeth together as well as the mean number of affected FPMs and incisors isolated were 2.9, 2.0 and 0.9, respectively.



Figur 16: Number of affected teeth among 110 individuals with MIH.

Altogether, 201 FPMs were affected in the 110 individuals with MIH. The numbers of affected FPMs showed that 48.2% of individuals had one affected FPM, 30.0% had two FPMs, 12.7% had three FPMs and 9.1% had four affected FPMs. Maxillary FPMs were 1.6 times more frequently affected than mandibular FPMs (P<0.001), and FPMs on the right side were 1.2 times more often affected than those on the left side (P=0.038) (Figure 17). Opacities were recorded in only 54.0% of these molars, while 24.3% had PEB. In addition, atypical restorations were found in 18.8% of the affected FPMs, and six teeth (3.0%) had been extracted due to MIH. The buccal

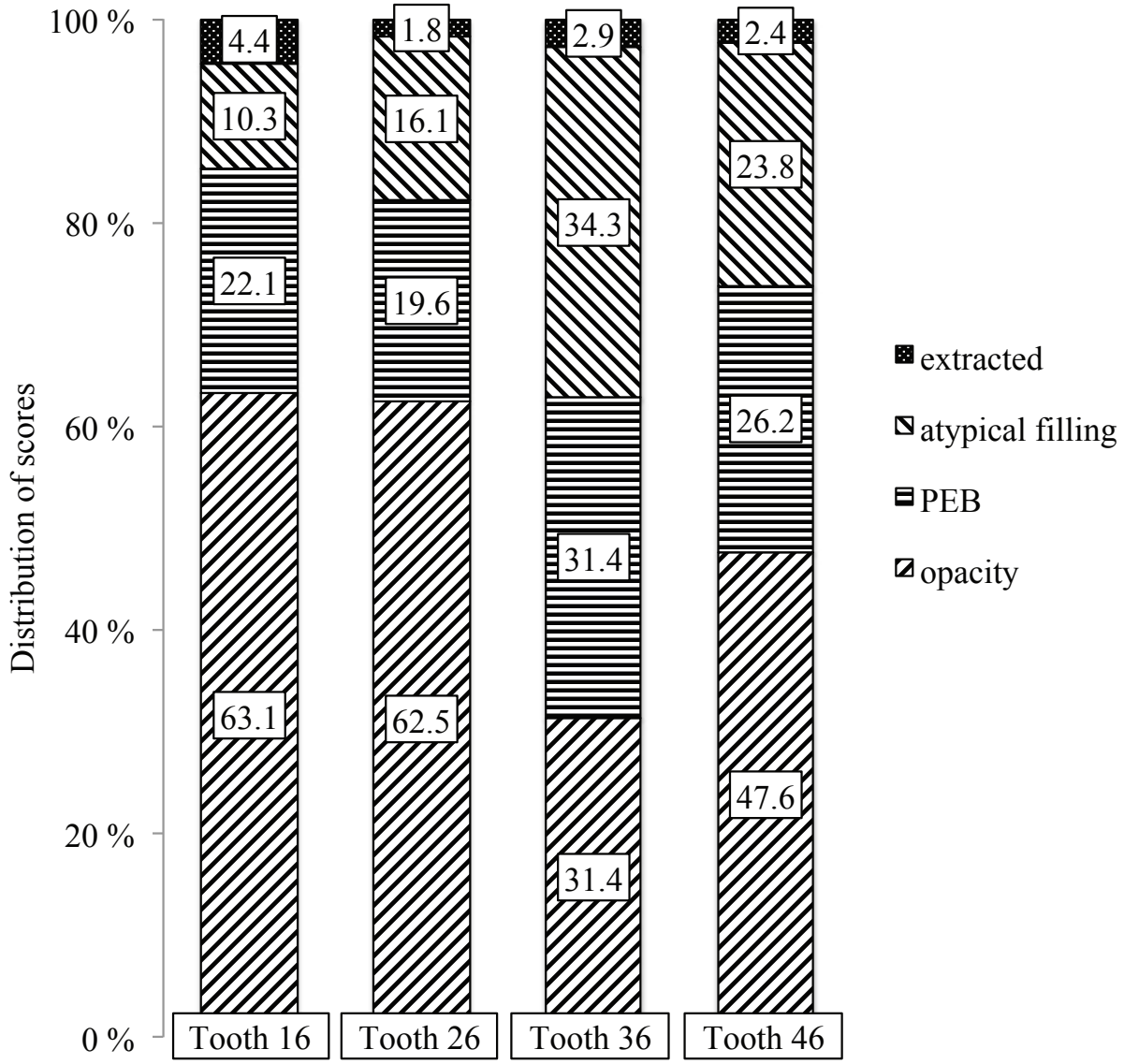


Figur 17: Proportion (%) and distribution of the affected MIH teeth among 110 individuals with MIH.

surfaces (78.6%) were most often affected, followed by the occlusal surfaces (39.3%), while the lingual surfaces (27.9%) were least frequently affected. According to Figure 18, more severe lesions (grade 2) were found in mandibular FPMs than in maxillary FPMs, 37.1% and 59.1%, respectively (P=0.002). Following the EAPD policy document, the registration of demarcated opacities at the lingual and palatal part of the crown is not recommended. In total, eight participants had solely an affection of the palatal (7 participants, 8 FPMs) or lingual (1 participant, 1 FPM) surface, increasing the overall prevalence from 12.8 to 13.9%.

Incisor involvement was recorded in 41.8% of the participants with MIH. A total of 32.8% had one or two affected incisors, and 9.0% had three to five affected incisors. Five was the maximum number of affected incisors in the same individual. Maxillary incisors were 2.5 times more often affected than mandibular incisors (P<0.001) (Figure 17). In the affected incisors, opacities were found on the buccal surfaces. No PEB was registered in incisors. In total, 91 affected incisors were registered, and no incisor was extracted due to MIH. The central incisors in the maxilla were more often affected than the lateral incisors, in contrast to the mandible, where the lateral incisors were most frequently affected.

Canines were involved in 22.8% of the individuals in the MIH group compared to 1.6% of those without MIH (P<0.001). All disturbances were localized in the incisal third, and in 10 out of 33 individuals, the cusp tip was affected. The number of affected canines in the MIH group ranged from 1 (15.5%) to 2 (7.3%), and maxillary canines were 2.3 times more often affected than mandibular canines (P=0.019). Of the 33 affected canines, no PEB was found in the mandible, but 13.0% had PEB or atypical fillings in the maxilla.



Figur 18: The distribution (proportion) and severity of the 201 affected FPMs in 110 individuals with MIH.

3.2. Paper II. Association between asphyxia during birth and the occurrence of MIH

Interexaminer and intraexaminer agreement for MIH diagnosis at the patient level, measured by Cohen's kappa, was in the range of 0.60–0.78 (mean 0.69) and 0.55–0.76 (mean 0.65), respectively.

The results of the joint decisions, based on the examination of the intraoral photographs, showed that the MIH prevalence in the asphyxia group was 29.4% and in the control group was 31.2% ($p = 0.79$). There was a slightly higher proportion of controls (5.7%) than cases (2.9%) who had an MIH sum score between 5 and 8 (Table 3), and there was no significant difference in the average severity score per tooth between the two groups ($p = 0.42$). Furthermore, there were no statistically significant differences in the distribution of affected index teeth or second primary

MIH score	Group		p-value
	Group 1 ($n = 68$)	Group 2 ($n = 157$)	
0	69.1	67.5	0.94
1–2	16.2	14.4	0.69
3–4	11.8	12.4	0.05
5–8	2.9	5.7	0.40
Total	100.0	100.0	

Table 3: Distribution of individuals according to MIH score in the study group (group 1) (Apgar score ≤ 5) and control group (group 2). The MIH score is cumulative score for all four FPMs in each individual. The scores for each tooth may vary from 0 (no MIH) to 2 according to severity. No statistically significant differences were observed between groups.

molars between the individuals within the groups (Table 4). An analysis of the medical records of the children in the study group who did not wish to participate showed that there was no increased morbidity during the first year of life in these children when compared with the children in the study group who participated.

There was no statistically significant difference in MIH prevalence between girls and boys in either the study group (p = 0.75) or the control group (p = 0.57).

Analyses of the self-reported health information (questionnaires) revealed that there was no statistically significant association with MIH and health-related variables between groups.

Affected tooth group	Group		p-value
	Group 1	Group 2	
Upper FPM	22.8	21.3	0.63
Lower FPM	11.0	18.5	0.13
Upper central incisors	5.3	6.1	0.61
Upper lateral incisors	3.5	3.8	0.54
Upper second primary molars	8.2	5.4	0.43
Lower central incisors	2.9	2.5	0.64
Lower lateral incisors	3.0	2.6	0.77
Lower second primary molars	8.2	4.1	0.08

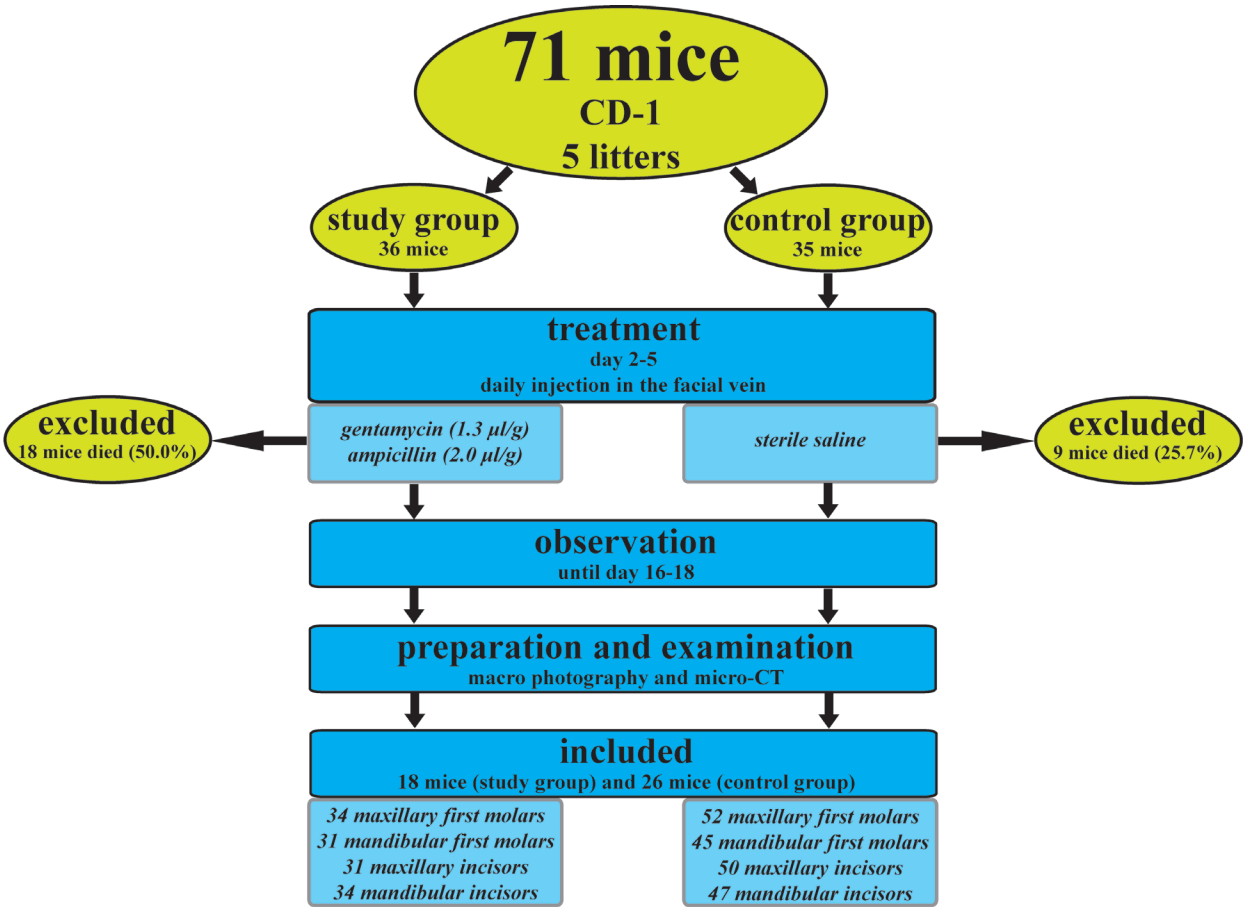
Table 4: Distribution of affected index teeth and second primary molars in individuals with an Apgar score ≤ 5 in the study group (group 1) compared to the control group (group 2). No statistically significant differences were observed between groups.

3.3. *Paper III. Effects of antibiotics on developing enamel in neonatal mice in vivo*

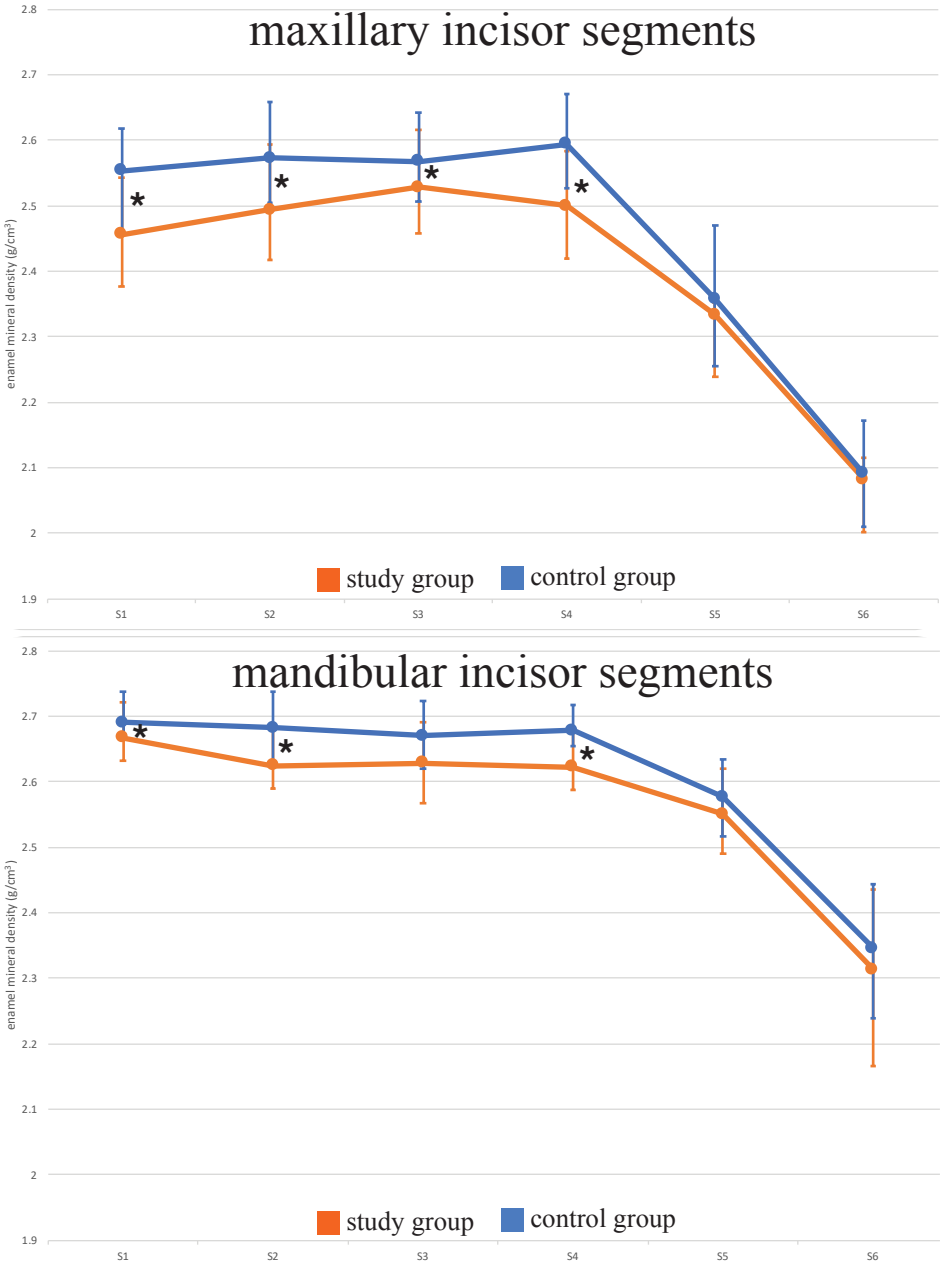
A flowchart of animals and teeth included in the final evaluation step of the study is presented in Figure 19. None of the mice died during or immediately after an injection; however, during the observation period until P16 to P18, 18/36 mice in the study group and 9/35 mice in the control group died (Figure 19). The mean (SD) age of the mice at the final time point was 16.9 (±0.78) days. Among the included mice, 14 first molars and incisors (7.9%) were excluded due to iatrogenic artifacts. In total, 18 mice from the study group with 65 first molars and incisors were included. In the control group, 26 mice with 97 first molars and incisors were evaluated (Figure 19).

When dividing incisors into six equal segments, the apical part of the enamel at approximately S5-S6 was less mineralized than the enamel closer to the incisal edge (Figure 20), which made the apical enamel more vulnerable during the dissection process, and in two cases, the most apical segments (S6) were excluded because of enamel fractures.

No enamel disturbances, such as demarcated opacities (white, yellow and brown color), hypoplasias or other enamel defects, were observed, neither in the study nor in the control group, by visual examination of macrophotographs (Figure 14). The enamel vol% in first molars was

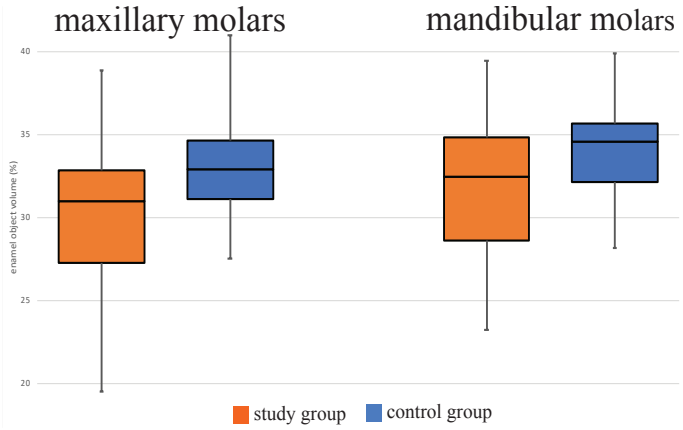


Figur 19: Flowchart of animals enrolled in the study.

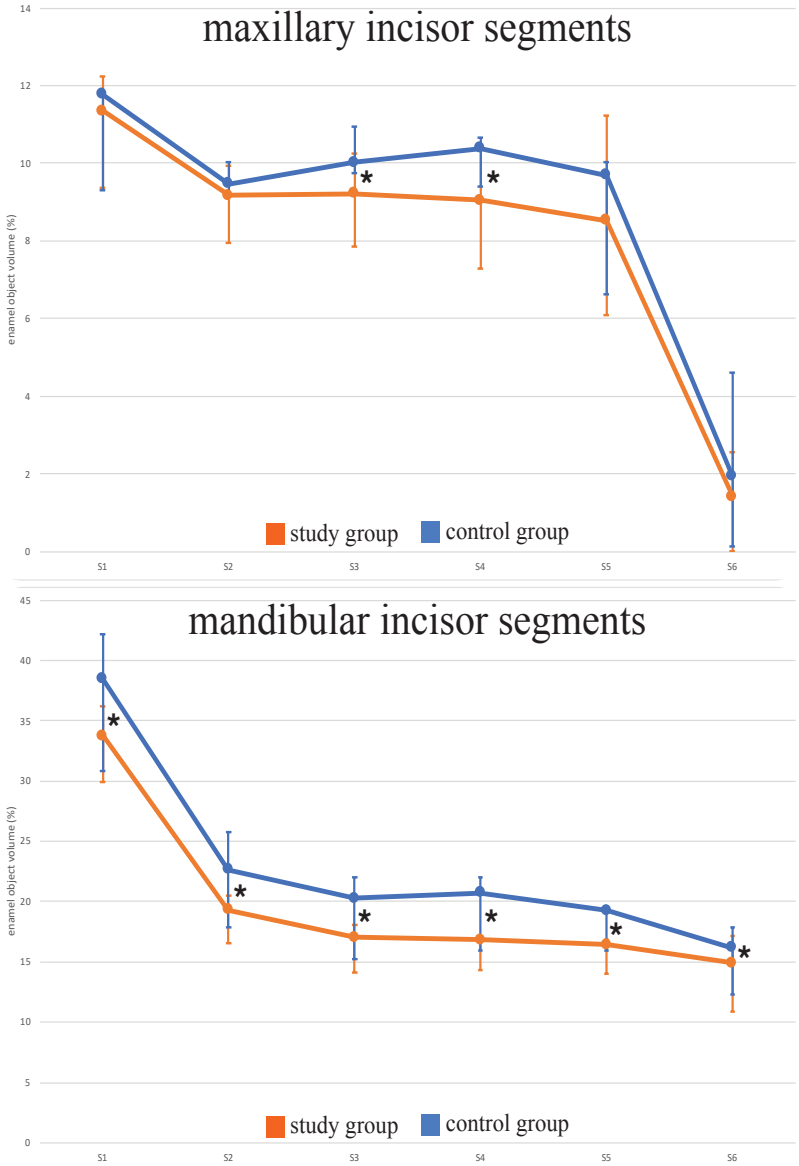


Figur 20: Median and interquartile range of enamel mineral density g/cm3 of maxillary and mandibular incisor segments (S1-S6); *significant difference between the study group and the control group ($p<0.05$).

significantly lower in the study group than in the control group in both the upper (30.9% vs. 32.7%; $p=0.004$) and lower (32.5% vs. 34.6%; $p=0.015$) jaws (Figure 21). The enamel vol% of the study group was significantly lower in mandibular incisors ($p<0.05$) in all segments (S1-S6). In the maxilla, two incisor segments (S3, S4) in the study group showed a significantly lower vol% than in the control group (Figure 22). The MD of the enamel from the study group was significantly lower ($p<0.05$) in four segments (S1-S4) from maxillary incisors than that from the control group. In mandibular incisors, the MD of the enamel was significantly lower in segments S1, S2, and S4 than in other segments (Figure 20). There was no significant difference between the MD of enamel from any of the first molars when comparing the study and the control groups.



Figur 21: Enamel object volume (%) in the maxillary and mandibular first molars; There were significant differences between study group and control group in both the maxillary (p=0.004) and the mandibular teeth (p=0.015).



Figur 22: Median and interquartile range of enamel object volume (%) in maxillary and mandibular incisor segments (S1-S6); *significant difference between the study group and the control group ($p<0.05$).

4. Discussion

4.1. Consideration of methodological aspects

This thesis consists of three studies with different study designs and study populations.

4.1.1. Paper I

Paper I was based on the health survey Fit Futures, and only adolescents born in 1994 were included in the survey. Most adolescents in Norway follow the normal school curriculum, but 16-year-olds not attending first-year upper-secondary schools were not a part of this survey. Reasons for missing one or more school years are many and varied. Factors such as sex, socioeconomic background, parents' education, earlier school accomplishments/achievement and too many missed school hours are mentioned in the literature (Markussen and Sandberg, 2010). Most of these factors may have no or little influence on the results regarding MIH. However, pupils with very special educational needs attend special classes or schools and are therefore not included in this study. Causes for special educational needs are mainly learning disabilities, which can be caused by certain circumstances, such as the mother becoming ill during pregnancy, problems during birth due to a lack of oxygen, genetic factors, illnesses such as meningitis and injury in early childhood. It has been speculated that adolescents with special educational needs are more frequently affected by MIH than healthy adolescents (Prokocimer et al., 2015), and a recent publication concluded that MIH is quite common in children with intellectual disabilities (Brzovic Rajic et al., 2021). The prevalence of MIH might be slightly underrepresented in the present study, since not all adolescents born in 1994 were invited to participate. On the other hand, the Fit Future participation rate (93%) was very high, ensuring good representativeness of the data.

Regardless of the attempts to ensure a high participation rate in this study, dropouts did occur. Possible causes for dropouts are many and varied. It has been reported that BMPs and DFA are more common in children with MIH than in controls (Jalevik and Klingberg, 2002). Therefore, it could be suspected that children with MIH would, for that reason, avoid dental examinations, especially when participation in an oral health research project is voluntary. However, in another study, there was no significant difference in DFA between children with severe MIH and children without MIH (Kosma et al., 2016). Since the main part of the Fit Futures health survey did not involve dental health, dropouts due to problems visiting dental services probably had no or only a negligible effect on the results.

Recommendations for studies on MIH, including the prevalence, severity, and number and distribution of affected teeth, were published in an EAPD policy document in 2003 (Weerheijm et al., 2003) and updated in 2010 (Lygidakis et al., 2010). Teenage participants are assumed to be a strength in MIH studies. The examination of participants at this age allows the assessment of all permanent teeth that could be affected by MIH, including canines. In addition to the prevalence

of MIH, the variability of the defects over time can be evaluated. The risk of underestimating the number and extent of MIH defects in older individuals due to caries lesions in affected areas or that PEB had been restored was not a problem in the present study. Norwegian dentists show positive attitudes towards minimally invasive dentistry, and Black's principles 'extension for prevention' are no longer tenable (Kopperud et al., 2016, Tyas et al., 2000). It is, however, recommended by William et al. (2006) that "All defective enamel should be removed to gain a better bonding of the adhesive material". Even if this recommendation is followed, it is assumed that MIH can be detected in most affected patients through the examination of the affection of other teeth. Experience during the scoring of the clinical photographs of the presented material suggested that it is unlikely that the presence of many restorations masks enamel disturbances. On the other hand, orthodontic treatment with fixed appliances can camouflage MIH lesions, and 68 participants (7.8% of the study group) had to be excluded for that reason.

While the EAPD policy document recommends the registration of demarcated opacities at the occlusal and buccal part of the crown (Lygidakis et al., 2010), the lingual and palatal surface of the crown were evaluated as well in this study, resulting in a slightly increased prevalence.

There is a substantial consensus to register the color of the opacities due to MIH, ranging from white, creamy or yellow to brownish. The colors of the opacities were not included in the analyses. Categorizing the clinical presentation of the opacities into these three groups (white, creamy or yellow to brownish) is difficult due to their heterogeneity, and the color of the opacities does not affect the severity classification of the affected tooth. The color is, however, of great importance for the characterization of the mechanical properties. Brown opacities have much lower mechanical resistance than white opacities (Fagrell et al., 2010).

Different gradations have been used regarding the minimal size of registered opacities. The current valid guidelines from the EAPD used in this study state that opacities less than 1 mm are not to be reported (Lygidakis et al., 2010), which is in line with the recommendation of developmental defects of enamel in general (FDI, 1992). One of the first MIH prevalence reports did not mention a minimal opacity size as an inclusion criterion (Koch et al., 1987), while in other publications, defects less than 2 mm are excluded (Jalevik et al., 2001b). A detailed list of judgment criteria for the diagnosis of MIH was published after a meeting of experts at the 6th Congress of the EAPD, but a specification of the criteria according to the size of opacities was lacking (Weerheijm et al., 2003). These earlier vague size definitions of opacities leading to different criteria may influence the comparability between various studies (Jalevik, 2010). In studies based on a clinical examination, a periodontal probe can be used to measure the size of an opacity, while in studies based on clinical photographs, the size of the opacity must be estimated, which could lead to misjudgments. In the present study, the difficulty of including opacities that were too small or excluded large enough opacities was solved by good calibration as well as

discussions leading to a joint decision.

The use of clinical photographs to diagnose MIH is an approved method even if most of the first prevalence studies from Scandinavia are based on data from clinical examinations (Alaluusua et al., 1996b, Jalevik et al., 2001a, Leppaniemi et al., 2001). A combination of both methods showed that there is a high agreement between clinical and photographic evaluations (Brogardh-Roth et al., 2011). Training and calibration of examiners using photographs may be easier, and standardized intraoral photographs may enable better comparability between different studies. It has been shown that the sensitivity and specificity of photograph-based detection of HSPM, which are comparable with those of MIH detection, are high, and the use of intraoral photographs is recommended in clinical practice as well as in epidemiological studies (Elfrink et al., 2009).

Observer variation is a factor that has to be considered when comparing results from different studies (Kopans, 2000). In the present study, these concerns were addressed using three observers who scored independently, and a final decision was made by consensus agreement.

In addition, the evaluation of clinical photographs by several experts is a more objective procedure and less invasive for the participant. The use of photographs facilitates randomization and blinding, limiting observer bias, which is important in a study such as this.

Other enamel disturbances must be differentiated from MIH. The most relevant differential diagnoses are dental fluorosis and amelogenesis imperfecta. Dental fluorosis is characterized by diffuse opacities that affect the teeth symmetrically (Soviero et al., 2009). Amelogenesis imperfecta affects the whole dentition, usually with opacities of the same degree. Teeth affected by MIH are rarely affected in the same degree (Mahoney and Morrison, 2009). The differentiation of MIH from other enamel disturbances is much easier when all permanent teeth can be evaluated. The risk of incorrect diagnosis is thereby minimized in the present age group.

4.1.2. Paper II

Paper II was based on a cross-sectional, case-control study. To reach an acceptable number of participants in the study group and enable the examination of all participants within a short period of time, ensuring consistent results and avoiding additional calibrations, a cohort born between 2004 and 2006 was chosen. All participants in the study could be examined at the age of 8–10 years. This age interval is favorable for the diagnosis of MIH. The age of 8 years is, according to the EAPD policy document, the best age for a cross-sectional study (Lygidakis et al., 2010). The examination of younger children raises the risk that not all FPMs are fully erupted. In addition, the risk of camouflaging MIH due to dental treatments/restorations increases with advancing age.

Since the Apgar score is based on a subjective grading, a calibration of the involved nurses and doctors might have been beneficial. The data were used retrospectively, and several of the participants with an Apgar score ≤ 5 after 5 minutes did not have a low score after 10 minutes. The use of the Apgar score after 10 minutes might have led to a study group including a higher

proportion of participants with a diagnosis of perinatal asphyxia. Due to a lack of sufficient registrations, such as metabolic acidosis, to confirm the diagnosis of perinatal asphyxia, the Apgar score after 5 minutes was used.

The dental examination of the participants in the study group was not part of a routine follow-up at the hospital. This dental examination required the additional cooperation of the parents, leading to a number of dropouts. Several of the participants in the study group had a high number of needed medical treatments, resulting in parents focusing on examinations with high benefits and the avoidance of additional examinations. It could be speculated that participants with a high grade of impairment declined participation to a higher degree than participants with a low grade of impairments.

The control group was recruited from a recall list. Since the recall interval is usually determined to be between 3 months and 24 months, patients with shorter recall intervals are likely overrepresented. Usually, patients with tooth development disturbances such as severe MIH have shorter recall intervals than patients with healthy teeth. Since this problem was not discovered before recruitment, an overrepresentation of MIH-affected participants could be expected.

The registration of MIH was done in line with the study in Paper I. The lower age of the participants might have affected their ability to cooperate, and to ensure the registration of defects due to MIH, a clinical examination was performed as well.

Differentiating MIH from other disturbances in tooth development was slightly more difficult than in the study in Paper I because of the lower age of the participants, in whom all permanent teeth may not have erupted.

4.1.3. Paper III

Paper III was based on results from an animal model using 71 neonatal mice. The mortality rate was not included in the calculation for the number of animals to be used. The reasons for the high mortality (27/71) are not fully understood. All animals died closely related to the treatment period, with an equal distribution within the first five days, which suggests that the injection technique itself, the contents of the injections used in both the study and control groups, or a combination of these factors may have been the cause of the unexpectedly high mortality. Antibiotics were administered intravenously, consistent with the treatment of human preterm infants diagnosed with sepsis. It could be speculated that the intravenous injection technique was too traumatic for some of the neonatal mice. A reported common problem when using an intravenous technique is the size and visibility of the target vein and the insertion the needle far enough into the vessels so that the bevel of the needle is completely surrounded by the vessel (Glascock et al., 2011). Both a Wee Sight Transilluminator and the addition of green dye to the solutions allowed visualization and were supposed to ensure a successful intravenous injection. The presented antibiotic administration technique is advanced, and mice at days 1-5 are small, so the risk for partial paravenous or

subcutaneous administration was obvious. This form of administration, however, was assumed to be likely effective. Therapeutic drug monitoring is possible by measuring the drug levels in blood, but this was not done to avoid additional burden for the animals.

The green food dye used in both groups to visualize and ensure proper injection resulted in a dramatically altered skin tone, which may have led to some pups being rejected by their mother (Glascock et al., 2011), which may partly explain the high mortality rate.

All these adverse side effects should be able to be detected shortly after injection and would cause the death of the animal. Although no obvious adverse effects were observed during or shortly after the injection, this evaluation was difficult since the mice did not show much activity the first days after delivery.

The mortality in the study group was twice as high as that in the control group. Since the difference in the mortality rate was so distinct, the dosage of the antibiotics used may have been responsible for the deaths in the study group. The potential for differences in pharmacokinetics between neonatal humans and mice remains uncertain (Warshawsky et al., 1981) and to our knowledge, there are no data available regarding the dosage or the combination of antibiotics used in neonatal mice.

All work with the animals was performed under the supervision of a well-trained animal keeper. The use of anesthesia prior to the intravenous injections was considered. However, the use of anesthesia prior to intravenous injections is normally not necessary in human neonates.

Several factors, which could not be addressed and examined in detail in this study, may have led to the high mortality rate. Several modifications to improve this protocol should be evaluated. Nevertheless, the presented protocol is considered a valid model for further research on MIH.

In enamel research, micro-CT is commonly accepted as a noninvasive technique to evaluate enamel density and volume. Hypomineralized enamel manifests as areas with lower density, while hypoplasia manifests as areas with a reduced volume. In contrast to analyses with a significantly higher resolution, such as scanning electron microscopy (SEM) or quantitative backscattered electron (qBSE) imaging, micro-CT is not dependent on the grinding of sections, and the grinding of longitudinal sections of mouse incisors is difficult (Sidaly et al., 2015b). Micro-CT enables a three-dimensional analysis of the enamel in the selected area (Fagrell et al., 2013), and defects can be detected independently of their location in the enamel. The use of an additional control group, using medication with tetracycline, would have enabled obtaining results to ensure the exact localization of the defect, and the number of sections included in the analyses would have been reduced. The analyses may have been done exclusively with only three sections: two sections representing the enamel formed right before and after the treatment with antibiotics and one section representing the affected enamel.

Severely hypomineralized enamel may be misinterpreted as dentine by micro-CT. This bias

was prevented by a visual examination of both clinical photographs and micro-CT images where misinterpretations could have been registered.

The difference in the MD of enamel between the study and control groups was less than 4% in the present study. The entire section, both sound and affected enamel, was analyzed. Due to the size of the human molars, affected enamel can be analyzed separately, which may result in greater differences (Fearne et al., 2004). The local temporary areas of hypomineralized enamel shown by Lyngstadaas et al. (1998) using SEM were not seen in the present study. It might be that micro-CT may not be sensitive enough to visualize these microscopic hypomineralizations (Neboda et al., 2017).

4.2. General discussion of the main results (Paper I)

4.2.1. MIH prevalence data

The present study reports the first MIH prevalence data in Norwegian adolescents. The prevalence of MIH was 13.9%, which is in line with most of the other Scandinavian studies. In Finland, Leppaniemi et al. (2001) reported an MIH prevalence of 19.3%, while in Sweden, Koch et al. (1987) found values varying from 3.6 to 15.4% depending on the age cohort included. Other Swedish studies reported prevalences of 18.4% (Jalevik et al., 2001a) and 16.0% (Brogardh-Roth et al., 2011). However, in a Danish study, a much higher prevalence (37.3%) was found (Wogelius et al., 2008), emphasizing the fact that the MIH prevalence is reported to vary considerably (Elfrink et al., 2015). The cause of this wide range in the reported prevalence of MIH is not yet understood, but geographical variations (Petrou et al., 2013, Wogelius et al., 2008) as well as differences between age cohorts (Koch et al., 1987, Kukleva et al., 2008) have been suggested. In general, the results from different studies might be difficult to compare due to the use of various indices and diagnostic criteria, the variability of analysis and the selection of participants (Hernandez et al., 2016). However, a meta-analysis based on studies from 43 countries yielded a mean (95% CI) prevalence of 13.1% (11.8-14.5%) (Schwendicke et al., 2018), which corresponds well to the results of the present study.

4.2.2. MIH and the affection of canines

Almost one-quarter of the MIH-affected individuals had one or more canines with signs of MIH. Studies reporting an affection of canines are rare. The great majority of studies focusing on the prevalence include 8–10-year-old participants (Elfrink et al., 2015). The median age (years) for the full eruption of permanent canines in the maxilla is 12.5 years, and in the mandible, it is 11.5 years (AlQahtani et al., 2010); this explains why the affection of canines is not reported by most studies. In the present study, maxillary canines were more than twice as often affected as lower canines. Mandibular canines showed only opacities, while PEB and atypical restorations occurred

in maxillary canines.

To our knowledge, only two publications have mentioned the affection of canines. Bhaskar and Hegde (2014) showed that 27.3% of MIH-affected children in India had hypomineralized canines and premolars, but they did not report the types of defects or the numbers of canines affected. The age of their study population ranged from 11 to 13 years. Dietrich et al. (2003) examined 2408 individuals aged 10 to 17 years and showed that 19.2% of the individuals with MIH had opacities on the cusps of canines. The frequency results of both of these studies were comparable with those of the present study, but no data about the distribution and MIH severity of the affected permanent canines were mentioned in these studies.

The presented data showed that a considerable proportion of canines were affected by MIH. This fact may be considered within research about the etiology of MIH, even if the affection of FPMs is the main focus. The tooth development of canines is delayed in comparison to FPMs. The calcification of FPMs starts in week 32 in utero, while the corresponding time is 4 months postpartum for canines. The crown of an FPM is completed at the age of 4 years and 3 months, compared to 6 years of age for the crown of a canine (Proffit et al., 2018, Schroeder, 1987).

Regarding the etiology, HSPM seems to be closely related to MIH. The calcification of the second deciduous molar starts at 18 weeks in utero, and the crown is completed at the age of 11 months (Schroeder, 1987). Consequently, the time period for the development of HSPM and MIH starts at 18 weeks in utero and is completed at the age of 6 years.

4.2.3. MIH data in more detail

The recorded difference in MIH prevalence between girls and boys (16.3% vs. 11.6%) was close to being statistically significant (P=0.054). The majority of international publications have not shown any statistically significant sex difference regarding MIH (Garcia-Margarit et al., 2014, Jasulaityte et al., 2007, Martinez Gomez et al., 2012, Preusser et al., 2007), nor have studies from Scandinavia (Leppaniemi et al., 2001, Wogelius et al., 2008). A few studies report that females are more frequently affected (Cho et al., 2008, Jeremias et al., 2013a), which is in line with the present study, where girls showed a somewhat higher prevalence than boys.

In the present study, the mean number of affected teeth (canines not included) was 2.9 among individuals with MIH, which is somewhat lower than that reported in other Scandinavian data, varying from 3.2 (Jalevik et al., 2001a) to 3.6 teeth (Wogelius et al., 2008). FPMs were more frequently affected than incisors, which is in line with previous reports (Jankovic et al., 2014, Kuhnisch et al., 2014a, Lygidakis et al., 2008b). The mean number of affected FPMs was 2.0, which corresponds well with other Scandinavian reports, in which the numbers ranged from 1.5 to 2.5 teeth (Brogardh-Roth et al., 2011, Wogelius et al., 2008). The mean number of affected incisors was 0.9, which is considerably lower than the 2.2 teeth affected reported in a Greek study (Lygidakis et al., 2008b).

Maxillary FPMs, incisors and canines were more often affected by MIH than mandibular FPMs, which is in accordance with most other studies (Leppaniemi et al., 2001, Martinez Gomez et al., 2012, Preusser et al., 2007). The upper right FPM has previously been reported to be the most frequently affected tooth (Lygidakis et al., 2008a, Martinez Gomez et al., 2012), which is consistent with the finding in the present study.

In agreement with previous studies, opacities were the most frequent enamel defect in MIH teeth (Allazzam et al., 2014, da Costa-Silva et al., 2010, Ghanim et al., 2011, Jasulaityte et al., 2007, Muratbegovic et al., 2007, Soviero et al., 2009, Wogelius et al., 2008). On the other hand, the frequencies of PEB and atypical restorations in FPMs were higher than those in most other reports (Ghanim et al., 2015, Petrou et al., 2015, Wogelius et al., 2008). The surface of some opacities may break down when the tooth has been exposed to the oral environment for a longer time, explaining the relatively high frequency of PEB in the present study.

The variations in the occurrence of MIH between homologous teeth is a characteristic that is not yet understood. This clinical experience is supported by the present results. Only 10.0% of participants showed a symmetrical distribution of enamel disturbances, which means that the same tooth on both sides was affected. Furthermore, the severity of the lesions varied between the corresponding teeth in most of these participants. The nonsymmetrical occurrence of enamel lesions could suggest that insults causing defective enamel are of short duration and affect ameloblasts in a critical phase (Fearne et al., 2004). In addition, considerable individual variation in the start of mineralization between homologous teeth (Sahlstrand et al., 2013) may cause nonsymmetrical MIH occurrence.

The occlusal and buccal surfaces were most often affected in FPMs, as well as the labial surface in incisors, also reported by Petrou et al. (2015). These surfaces have thicker enamel than the lingual/palatial surfaces in FPMs (Lygidakis et al., 2010). The thicker the enamel is, the longer the formation period, which increases the possibility of enamel disturbances (Lygidakis et al., 2010). Another interesting phenomenon of MIH is that the cervical third of the tooth is usually not affected (Jalevik and Noren, 2000, Preusser et al., 2007), which might be related to the thin enamel in this area. Further studies are needed to describe the location of defects on the enamel surface and to relate these findings to enamel thickness and the duration of amelogenesis.

4.3. General discussion of the main results (Paper II)

4.3.1. MIH and Apgar scores ≤5 at 5 min

In the present study, it was tested whether Apgar scores ≤5 at 5 min increased the risk of MIH development. However, no statistically significant difference in the prevalence of MIH between the study group and the control group was shown. Furthermore, the number of affected FPMs or the severity of MIH in affected teeth did not differ between groups.

The prevalence of MIH corresponds well with findings from some other studies within Scandinavia (Wogelius et al., 2008), (Jalevik et al., 2001a, Jalevik et al., 2018). The prevalence of MIH in this study, however, was considerably higher than the prevalence reported in Paper I, although the same recording methods were used. As mentioned before, the cause of this wide range in the reported prevalence of MIH is not yet understood, but geographical variations (Petrou et al., 2013, Wogelius et al., 2008) as well as differences between age cohorts (Koch et al., 1987, Kukleva et al., 2008) have been suggested.

There was no significant difference in the prevalence of MIH between sexes in this study, which is in line with most MIH studies from Scandinavia (Leppaniemi et al., 2001, Wogelius et al., 2008) as well as international publications (Jasulaityte et al., 2007, Martinez Gomez et al., 2012, Preusser et al., 2007). The relative distribution of MIH between tooth groups and the upper and lower jaw is consistent with the findings that have been reported in Paper I and by (Lygidakis et al., 2008a).

4.3.2. Hypoxia

Human cells respond to reduced oxygen availability. The reason for this response, with numerous adaptive mechanisms, is to facilitate cell survival by maintaining cellular activity at a minimum level of oxygen. Normally, when the oxygen level is restored, the hypoxic response will cease (Chen et al., 2020). Whether this damage will be temporary or permanent depends on the affected tissue, as well as on the duration and the level of oxygen. Since a disturbance in amelogenesis is not reversible, any damage during hypoxia might be visible forever.

Cellular adaptations to hypoxia rely on the transcription factor hypoxia-inducible factor 1-α (HIF1-α). Studies show that tuftelin, an acidic protein that plays an important role in tooth development and enamel mineralization, is also involved in adaptation to hypoxic conditions (Shilo et al., 2019). Tuftelin is found in the extracellular developing enamel, and it is concentrated at the dentin-enamel junction (DEJ), where the mineralization of the enamel commences (Deutsch et al., 1995). The failure of these adaptation mechanisms during hypoxia results in cellular dysfunction and can lead to irreversible cell damage. Furthermore, it may be speculated that compensatory mechanisms such as increased expression of HIF1-α, which is shown to be upregulated in ameloblast cells following hypoxia (Sidaly et al., 2015a), may be the reason why MIH does not develop in patients with mild hypoxia.

Birth asphyxia is associated with metabolic acidosis, which is measured in arterial umbilical cord blood. A close association between Apgar scores and rates of metabolic acidosis exists. In 2004-2006, umbilical blood gases were usually not analyzed in Norway. Therefore, an Apgar score ≤5 at 5 min was used as an entry criterion for further neontological diagnosis/treatment at that time. Even if an association between the Apgar score and metabolic acidosis exists, it cannot be assumed that all participants with an Apgar score ≤5 at 5 min had severe hypoxia. Thus, the hypoxia of the

study group might not have been severe enough to induce changes in the ameloblasts to cause MIH.

Several studies have suggested that hypoxia may adversely affect ameloblast function (Beentjes et al., 2002, Jalevik, 2001, Johnsen et al., 1984, van Amerongen and Kreulen, 1995). The results from the present clinical investigation were not consistent with the findings reported in an animal study (Sidaly et al., 2015b), where a short episode of induced severe hypoxia in adult mice left its mark on mouse incisors in the form of enamel defects.

4.4. General discussion of the main results (Paper III)

The visual examination of the teeth in the study group did not show any opacities. Therefore, standardized sections were defined to evaluate the effect of the antibiotics on amelogenesis. The analyses were performed by comparing each section with the neighboring section. The results demonstrated that therapeutic doses of the antibiotic combination of ampicillin and gentamicin affected both the quality and quantity of enamel in mice. It was demonstrated that the use of these antibiotics reduced incisor MD values. Similar alterations are observed in teeth affected by MIH. The enamel volume was reduced as well, which is consistent with MIH. In MIH-affected human molars, the MD value is reported to be approximately 20% lower than that in clinically sound teeth, while the quantity of enamel is not affected (Fearne et al., 2004).

In mouse molars, the secretion of enamel (7 μm/d) takes approximately 15 days, with a final enamel thickness of approximately 100 μm (Lyngstadaas et al., 1998). In the present study, the enamel volume of the first molars was significantly reduced in the mice that received the drugs. A reduced volume of enamel leads to hypoplasia, indicating that the secretory phase of amelogenesis is affected. This is consistent with results showing thinner enamel in rats treated with amoxicillin during the secretory stage of amelogenesis (de Souza et al., 2016).

The major odontogenic signaling interactions are almost identical among various mammalian species, including mice and humans. Mouse models may help to contribute to the understanding of tooth developmental defects such as MIH (Fleischmannova et al., 2008). The use of mice as model organisms to study human biology is common. Nevertheless, mouse age must be determined in relation to human age (Dutta and Sengupta, 2016), which is crucial in research within MIH, where mouse amelogenesis takes place in a very short period of time. Until now, no animal model of MIH has been established, and spontaneous effects on mouse incisors and molars in line with MIH have never been reported.

Studies of the effect of antibiotics in mice, focusing on the effect on amelogenesis, are rare and show conflicting results. In contrast to the present results, amoxicillin and ampicillin used in embryonic mouse molars in vitro produced an increased enamel thickness (Laisi et al., 2009). The authors speculated that amoxicillin interferes with the function of the ameloblasts by delaying the

differentiation of ameloblasts into secretory cells. The altered pattern of amelogenesis due to the early use of amoxicillin within the initiation phase of amelogenesis or the enamel secretion rate could explain the hypomineralization in MIH (Laisi et al., 2009). While a significantly thinner enamel in rats treated pre- and postnatally with amoxicillin has been demonstrated by de Souza et al. (2016), no effect on enamel after amoxicillin treatment in rats was shown by Kumazawa et al. (2012). However, even if no effect on enamel thickness was shown in rats treated prenatally with amoxicillin, dose-dependent enamel hypomineralization has been observed (Gottberg et al., 2014). This result was confirmed in a study on mice by Mihalas et al. (2016). The authors concluded that chronic exposure to amoxicillin in doses used in humans led to hypomineralized enamel. It has been suggested that amoxicillin affects the expression of matrix metallopeptidase 20 (MMP20), which has an important role in the degradation and removal of enamel proteins (Sahlberg et al., 2013).

Studies on mice have shown that in the first molars, ameloblasts at the tips of intended cusps enter the secretory phase just after birth, while at day two, crown mineralization advances by involving more ameloblasts in enamel production. On the fourth day, dental crown morphogenesis is completed (Lungova et al., 2011). Other studies have shown different results. According to Miyata et al. (2007), the first molar of mice at day six shows the mid- or early stage of crown formation. However, different strains of mice are used in these studies, and differences in timing should be expected. Lungova et al. (2011) described a partially erupted first molar on day 16. At the same time, all first molars fully erupted at day 18 in the present study. Medical treatment was performed from days 2 to 5, and the maturation stage of enamel morphogenesis could possibly be affected but probably not the initial phase of amelogenesis.

In contrast to incisors, mouse molars present a rapidly developing model for odontogenesis in human molars. The mean formation time of the crown in human FPMs is more than three years (Reid and Dean, 2006), while it is approximately three weeks in mice (Lungova et al., 2011). This quick sequence of stages in mice may make a direct comparison difficult (Fejerskov, 1979), especially for the study of the transition stage (between secretion and maturation), which has been assumed to be the most vulnerable stage for the affection of ameloblasts (Fearne et al., 2004).

Mice have just a single dentition, probably the equivalent to human deciduous teeth (Tucker and Sharpe, 2004). Although the present study aimed to explore MIH in the human permanent dentition, similar defects exist in the primary dentition (Elfrink et al., 2012). It is not clear whether the human conditions of MIH or HSPM can be reproduced in mice. Additionally, the extrapolation of findings from the animal model to humans should be taken with care. Animal research is ideal for studying genetic defects or to characterize genetic-environmental interactions (Muthanandam et al., 2020). The limitations are that the genetic aspects within MIH are suspected but not clear (Vieira, 2019a).

5. Conclusion

The prevalence of MIH was 13.9% in the presented cross-sectional health survey of 16-year-olds from northern Norway. This prevalence as well as the distribution of affected teeth and the severity of MIH were consistent with previous Scandinavian reports. The distribution pattern showed that one in four participants with MIH had at least one affected canine. Further studies are needed to describe the location of defects on the enamel surface and to relate these findings to enamel thickness and the duration of amelogenesis.

No association between a low Apgar score at 5 min and the appearance of MIH or the number of affected FPMs was shown in the presented cross-sectional, case-control study of 8- to 10-year-old children. A low Apgar score is not necessarily associated with severe hypoxia, which is supposed to be an etiological factor for MIH. In further MIH studies, a low Apgar score should be associated with other clinical markers of asphyxia, such as acidosis.

Based on the results of animal research using neonatal mice, intervention with the high-dose antibiotics gentamycin and ampicillin influenced the development of molars and incisors. The reduced enamel MD and volume of first molars and the erupted part of incisors are likely to have been caused by antibiotics. The analysis of teeth in neonatal mice with micro-CT could be a valid model for further research on MIH. As research on the effect of antibiotics on enamel development did not show conclusive results, further research is needed.

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Papers I-III

Paper I

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Canines are affected in 16-year-olds with molar–incisor hypomineralisation (MIH): an epidemiological study based on the Tromsø study: "Fit Futures"

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Abstract
Aim This was to determine the prevalence, distribution of affected teeth and severity of MIH in adolescents from Northern Norway.
Methods It was part of a cross-sectional health survey Fit Futures including 16-year-olds from two neighbouring municipalities, Tromsø and Balsfjord.
Results The prevalence of MIH was 13.9 % (110 of 794). The maxillary first permanent molars (FPMs) were 1.6 times more frequently affected than in the mandible ($P < 0.001$). The FPMs on the right side were 1.2 times more often affected than the FPMs on the left side ($P = 0.038$). The maxillary incisors were 2.5 times more often affected than the incisors in the mandible ($P < 0.001$). The proportions of participants whose canines and incisors were involved were 22.8 and 41.8 %, respectively. Altogether 201 FPMs were affected; 54.0 % of these had opacities only, 24.3 % had posteruptive breakdown (PEB), 18.8 % had atypical restorations, and 3.0 % had been extracted due to MIH. The buccal surfaces were most often affected in FPMs. More severe lesions were found in the mandibular FPMs compared with the maxillary FPMs ($P = 0.002$). In the lower canines, only opacities were recorded, while in the upper jaw 13.0 % of the affected canines showed PEBs. The distribution of MIH in the dentition was not symmetrical.
Conclusion The prevalence of MIH (13.9 %) in the study population of 16-year-olds from Northern Norway is consistent with previous Scandinavian reports. The distribution pattern shows that one participant in four with MIH had at least one affected canine. Further studies are needed to describe the localisation of defects on the enamel surface and to relate these findings to enamel thickness and the duration of amelogenesis.

Keywords Molar–incisor hypomineralisation · Prevalence · Epidemiology · Norway

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Introduction

The prevalence of molar–incisor hypomineralisation (MIH) varies considerably between different regions ranging from 2.4 % in Germany and Bulgaria (Dietrich et al. 2003; Kukleva et al. 2008) to 40.2 % in Brazil (Soviero et al. 2009). In Nordic countries, the prevalence ranges from 17.0 % in Finland (Alaluusua et al. 1996a) to 37.3 % in Denmark (Wogelius et al. 2008), but so far no study has been conducted in Norway.

The most frequently affected teeth in MIH are first permanent molars (FPMs) and permanent incisors, which are mineralised around the time of birth. The second primary molars and the tips of the permanent canines can also be involved occasionally (Weerheijm et al. 2001, 2003).

MIH was so named by Weerheijm et al. (2001), and judgment criteria for diagnosis were defined in 2003 (Weerheijm et al. 2003) and modified in 2010 (Lygidakis et al. 2010), but the condition had already been described many years ago by Koch et al. (1987). According to the recommendations of the European Academy of Paediatric Dentistry (EAPD) (Lygidakis et al. 2010), the best age for a

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cross-sectional study of MIH would be eight years. To evaluate the clinical variability of the enamel disturbances over time (Lygidakis et al. 2010), a longitudinal study design with examinations from the age of 6 up to 12 years is recommended. Kühnisch et al. (2014) recommended including 14- to 16-year-olds, allowing more complete monitoring of MIH (Jälevik 2010; Lygidakis et al. 2010; Kühnisch et al. 2014).

To our knowledge, only two publications (Dietrich et al. 2003; Bhaskar and Hegde 2014) have mentioned that permanent canines were affected in some individuals, but no data about the distribution of affected permanent canines are available. The median age of full eruption of maxillary permanent canines (both sexes) was 12.5 years and for mandibular permanent canines, 11.5 years (AlQahtani et al. 2010).

One feature of MIH typically observed is its non-symmetricity. It seems that this has not hitherto been thoroughly reported in the literature.

The affected enamel in MIH teeth has a tendency to accumulate more severe defects over time due to posteruptive breakdown (PEB) of hypomineralised enamel (Weerheijm et al. 2001).

The present study aimed (1) to report on the prevalence of MIH in Norwegian adolescents, (2) to examine the distribution of the affected teeth and (3) to describe the severity of the enamel disturbances 5–10 years after eruption.

Materials and methods

In 2010–2011, all first-year upper secondary school students in the two neighbouring municipalities in Northern Norway, Tromsø and Balsfjord, were invited to join the cross-sectional health survey *Fit Futures* with an attendance rate of 92.9 % (Winther et al. 2014). All participants gave written informed consent. Participants aged 16 years and above signed at the study site, while younger participants brought written permission from their guardian. In the present study, only individuals born in 1994 (380 girls and 414 boys) were included (Fig. 1). The Norwegian Data Protection Authority and The Regional Committee of Medical and Health Research Ethics (reference number 2009/1282 and 2011/1702/REK nord) approved the study in July 2010 and October 2011, respectively.

As a part of the clinical examination, eight photographs (Canon EOS 60D; Canon 105 mm; Sigma EM-140 DG) were taken in the following order: the buccal surfaces of the teeth in the first and fourth quadrant (#1), the corresponding surfaces in the second and third quadrant, the buccal surfaces of the maxillary and mandibular anterior teeth (#3), the occlusal surfaces of the upper teeth (#4 & 5)

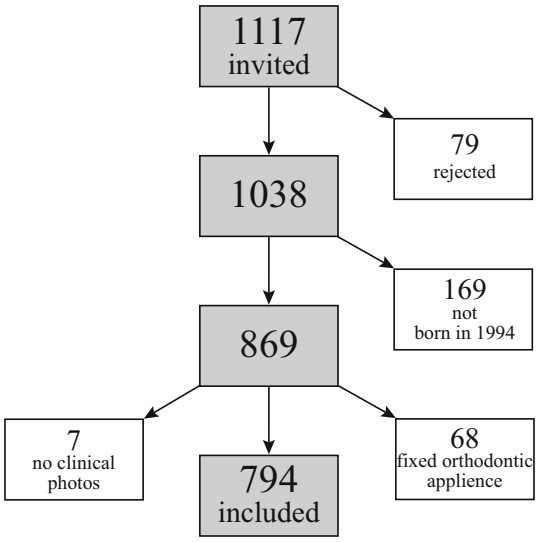

Fig. 1 Participant inclusion flow chart

and lower teeth (#6 & 7) and the palatinal surfaces of the upper anterior teeth (#8).

The clinical photographs of the 794 adolescents were shown on a flat screen in a room with indirect, standardised lighting and examined independently by three experienced dentists (examiners AS, ABT, KS). In line with the EAPD guidelines of MIH (Lygidakis et al. 2010), the buccal, occlusal and palatinal/lingual surfaces of all FPMs were examined as well as the labial surfaces of all central and lateral incisors and canines. Characteristics such as opacities (white cream/yellow-brown colour), PEB, atypical restorations and extractions judged as being due to MIH were recorded. Opacities >1 mm were registered (Lygidakis et al. 2010). The examiners recorded individually and independently. A joint score was decided for each recording, and a consensus was reached through discussion when individual scores differed. Classification of MIH-affected teeth and surfaces was based on the most severe diagnosis recorded (opacity < PEB < atypical restoration).

Affected MIH teeth were given a dichotomous score classifying the lesions as *mild* or *severe*. Surfaces or teeth with opacities only were defined as *mild* (grade 1). Surfaces or teeth with PEB, atypical fillings and teeth that had been extracted were defined as *severe* (grade 2). If an opacity and PEB or restoration occurred on the same surface, it was scored as *severe* (Lygidakis et al. 2010).

To calculate the intra- and inter-examiner agreement, a re-examination was performed one month later. The examiners repeated the registrations of 10 % ($n = 11$) of cases randomly selected with MIH diagnoses and 10 % ($n = 68$) without MIH diagnoses. The cases were mixed and "blinded" before the re-examination. The codes which

were used in the kappa calculations (tooth level) were: 0 = MIH free and 1 = MIH affected.

Statistical analysis

The data were analysed using the SPSS package version 21.0 (IBM SPSS Inc., Chicago, IL, USA). Inter-observer analyses (kappa statistics) were performed with MedCalc version 13 (MedCalc Software, Ostend, Belgium).

Results

The mean (SD) age of the participants was 16.6 (±0.33) years (range 15.8–17.3 years). The inter-observer variation is reported in Table 1. The three examiners (O1–O3) had the following intra-observer variation expressed as kappa (95 % CI) 0.88 (0.80–0.95), 0.89 (0.81–0.96) and 0.86 (0.78–0.94).

The prevalence of MIH was 13.9 % (110 of 794 participants). Girls were more often affected than the boys (16.3 vs. 11.6 %; $P = 0.054$). The mean numbers of affected index teeth, FPMs and incisors were 2.9, 2.0 and 0.9, respectively, in participants with MIH. In about half of the participants with MIH (50.9 %), the number of affected teeth was limited to one or two teeth. About a quarter (27.3 %) had three or four affected teeth, while 21.8 % had five or more affected teeth (Fig. 2).

Only one FPM was affected in 48.2 % of individuals with MIH, while 30.0 % had two, 12.7 % had three, and 9.1 % had four affected FPMs. Maxillary FPMs were 1.6 times more frequently affected than mandibular ones ($P < 0.001$), and the FPMs on the right side were 1.2 times more often affected than those on the left side ($P = 0.038$) (Fig. 3).

Incisor involvement was recorded in 41.8 % of the participants with MIH; 32.8 % had one or two affected incisors, and 9.0 % had three to five incisors affected. Five was the maximum number of affected incisors in the same individual. The maxillary incisors were 2.5 times more often affected than the mandibular incisors ($P < 0.001$) (Fig. 3).

Canines were involved in 22.8 % of the individuals in the MIH group compared to 1.6 % of those without MIH ($P < 0.001$). All disturbances were localised in the incisal third of the canines, and in 10 out of 33 (30.3 %), the cusp tip was affected. The number of affected canines ranged from 1 to 2 among individuals with MIH, while none of the participants without MIH had more than one canine with enamel disturbance. The maxillary canines in the MIH group were 2.3 times more often affected than mandibular ones ($P = 0.019$) (Fig. 3). In participants without MIH, a total of 11 out of 2736 canines (0.4 %) were registered with enamel disturbances at the cusps of the crown.

The mean DMFS score was higher in participants who did not have MIH (6.2 surfaces) compared with those with MIH (5.6 surfaces) ($P = 0.331$).

Among the participants with MIH, 1.8 % of all FPMs had been extracted, 1.4 % (6 teeth) due to MIH and 0.4 % for other reasons. Among individuals without MIH, 0.3 % of all FPMs had been extracted. Altogether 201 FPMs were affected in the 110 individuals with MIH.

Opacities only were recorded in 54.0 % of these molars, while 24.3 % had PEB. In addition, atypical restorations were found in 18.8 % of the affected FPMs, and six teeth (3.0 %) had been extracted due to MIH (Fig. 4).

The buccal surfaces (78.6 %) were most often affected in FPMs, followed by the occlusal surfaces (39.3 %), while the lingual surfaces (27.9 %) were least frequently affected (Table 2). In the maxillary FPMs, the occlusal and lingual surfaces were more frequently affected compared with the lower FPMs (Table 2). More severe lesions (grade 2) were found in the mandibular FPMs compared with the maxillary FPMs, 37.1 and 59.1 %, respectively ($P = 0.002$) (Fig. 4).

In the affected incisors, the opacities or PEBs were found on the buccal surfaces. One exception was an individual who had palatal opacities in both maxillary central incisors and unaffected enamel on the buccal surfaces. In total, 91 affected incisors were registered and no incisor was extracted due to MIH. The proportion of PEBs and atypical fillings was higher in the maxillary incisors compared with the corresponding lower teeth (16.9 vs. 11.5 %; $P = 0.52$).

Table 1 Inter-observer O1–O3 variation, kappa (95 % CI)

	Observer	
Observer	O2	O3
O1	0.92 (0.89–0.94)	0.91 (0.89–0.93)
O2		0.99 (0.98–1.0)

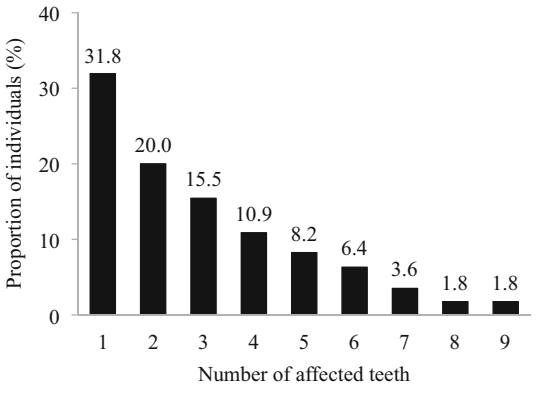

Fig. 2 Number of affected teeth among 110 individuals with MIH

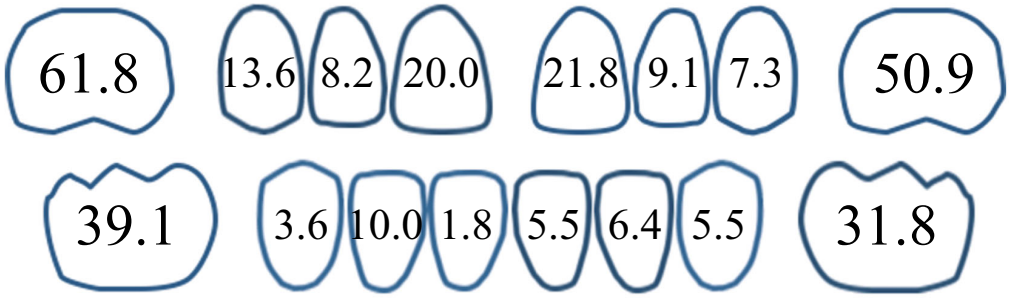

Fig. 3 Proportion (%) and distribution of the affected MIH teeth among 110 individuals with MIH

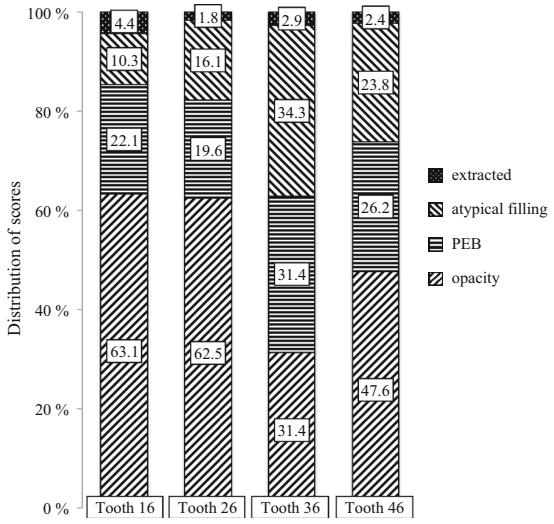

Fig. 4 The distribution (proportion) and severity of the 201 affected FPMs in 110 individuals with MIH

The central incisors in the maxilla were more often affected than the laterals, in contrast to the mandible where the laterals most frequently were involved (Fig. 3). Of the affected 33 canines in MIH group, no PEB was found in lower jaw, but 13.0 % had PEBs or atypical fillings in upper jaw.

Out of 110 participants with MIH, 11 cases (10.0 %) displayed bilaterally symmetrical distribution of enamel disturbances; the same teeth on both sides were affected. However, the severity varied between the corresponding teeth in the majority of these cases.

Discussion

The individuals reported on in the present study were older than in other epidemiological studies of MIH with the exception of that of Dietrich et al. (2003) who included 16-year-olds and 17-year-olds in their survey. The age of the included patients in the present study allowed the extent to which permanent canines were affected to be studied. Altogether 794 16-year-olds were included, and the prevalence of MIH was 13.9 %. Almost one-quarter of the MIH-affected individuals (22.8 %) had one or more canines with signs of MIH and significantly more frequent than among the non-affected individuals.

In Finland, Leppäniemi et al. (2001) reported a MIH prevalence of 19.3 %, while in Sweden, Koch et al. (1987) found values varying from 3.6 to 15.4 % depending on the age cohort included. Other Swedish studies reported prevalences of 18.4 % (Jälevik et al. 2001) and 16.0 % (Brogardh-Roth et al. 2011). However, in a Danish study a considerably higher prevalence (37.3 %) was found

Table 2 Distribution of enamel defects due to MIH on three index surfaces in affected FPMs

Tooth (*n*)	Tooth 16 (68)			Tooth 26 (56)			Tooth 36 (35)			Tooth 46 (42)		
Index surface	B	O	L	B	O	L	B	O	L	B	O	L
Number of affected surfaces	52	23	26	40	24	19	30	15	5	36	17	6
Grade 1: mild lesions (opacities)	43	7	13	30	11	6	10	7	1	17	5	3
Grade 2: severe lesions	9	16	13	10	13	13	20	8	4	19	12	3
PEB	4	9	8	5	6	8	10	2	3	12	2	0
Atypical restoration	2	4	2	4	6	4	9	5	0	6	9	2
Extracted		3			1			1			1	

PEB Posteruptive breakdown, *B* buccal, *O* occlusal, *L* lingual

(Wogelius et al. 2008). These reports from Nordic countries and international studies emphasise the fact that MIH prevalence varies considerably (Elfrink et al. 2015). The cause of the wide range in reported prevalence of MIH is not yet understood, but geographical variations (Wogelius et al. 2008; Petrou et al. 2013) as well as differences between age cohorts (Koch et al. 1987; Dietrich et al. 2003; Kukleva et al. 2008) have been suggested. Participation in the present study was high (92.9 %), which strengthens its internal validity.

Different diagnostic criteria, varying numbers of examiners and different types of examination (clinical examination vs. photograph evaluation) can lead to incomparable data. Most Scandinavian studies are based on clinical examinations (Alaluusua et al. 1996b; Jälevik et al. 2001; Leppäniemi et al. 2001; Wogelius et al. 2008), while Brogardh-Roth et al. (2011) used both clinical examinations and clinical photographs. In the present study, clinical photographs and the EAPD criteria (Lygidakis et al. 2010) were used. Only opacities greater than 1 mm were registered. In other Scandinavian studies, opacities smaller than 2 mm were excluded (Jälevik et al. 2001; Leppäniemi et al. 2001; Brogardh-Roth et al. 2011) or else the size of the defects was not a stipulated inclusion criterion (Koch et al. 1987).

Observer variation is a factor that has to be considered when comparing results from different studies (Kopans 2000). In the present study, these concerns have been addressed by using three observers who scored independently and a final decision was made by consensus agreement.

Clinical photographs, as a basis for MIH examinations, have been used in three studies by Elfrink et al. (2009, 2012, 2013). It has been shown that the sensitivity and specificity of photograph-based detection of deciduous molar hypomineralisation (DMH) using the adapted MIH criteria were high. The inter- and intra-observer reliabilities for DMH were good to excellent. The authors suggested that intra-oral photographs may be used in clinical practice and in large epidemiological studies (Elfrink et al. 2009).

The recorded difference in MIH prevalence between girls and boys (16.3 vs. 11.6 %) was close to statistical significance ($P = 0.054$). In previous MIH studies from Scandinavia (Leppäniemi et al. 2001; Wogelius et al. 2008), and in most international publications (Jasulaityte et al. 2007; Preusser et al. 2007; Martínez Gómez et al. 2012; Garcia-Margarit et al. 2013), no statistically significant gender difference had been found. A few studies reported that females were more frequently affected. Jeremias et al. (2013) found that the prevalences among girls and boys were 62 vs. 38 %, while Cho et al. (2008) showed a female-to-male ratio of 1.2:1.

In the present study, the mean number of affected teeth (canines not included) was 2.9 among individuals with MIH. This was somewhat lower than in other Scandinavian data, in which this number varied from 3.2 (Jälevik et al. 2001) to 3.6 teeth (Wogelius et al. 2008). The present study showed that FPMs were more frequently affected than incisors (Fig. 3), which is in line with previous reports (Lygidakis et al. 2008b; Kühnisch et al. 2014; Jankovic et al. 2014). The mean number of affected FPMs was 2.0, which corresponds well with other Scandinavian reports in which the numbers range from 1.5 to 2.5 teeth (Wogelius et al. 2008; Brogardh-Roth et al. 2011). The mean number of affected incisors was 0.9. This was considerably lower than the 2.2 teeth reported in a Greek study (Lygidakis et al. 2008b). In the present study, almost one quarter of the MIH-affected individuals had at least one affected canine in comparison with 1.6 % in the group without MIH ($P < 0.001$). There are only two reports mentioning MIH-affected permanent canines (Dietrich et al. 2003; Bhaskar and Hegde 2014), because the study populations are usually too young to have erupted canines. Bhaskar and Hegde (2014) showed that 27.3 % of MIH-affected children in India had hypomineralised canines and premolars, but did not report the types of defect or numbers of canines affected. The age of their study population ranged from 11 to 13 years. Dietrich et al. (2003) examined 2408 individuals aged 10–17 years and showed that 19.2 % of the individuals with MIH had opacities on the cusps of the canines. The present study showed that the maxillary canines were more than twice as often affected than those in the mandible and the opacity-to-PEB/atypical restorations ratio was 6.7:1. The lower canines showed only opacities; all PEB/atypical restorations occurred in maxillary canines.

The participants in the present study were older than in most other MIH studies (mean age 16.6 years), and affected teeth had been in occlusion for 5–10 years. Tooth wear and previous dental treatment could have masked the prevalence of MIH. This could lead to an underestimation of MIH, but this age group gave the opportunity to evaluate the permanent canines. However, the DMFS value was low in the MIH group and not statistically significantly different from the non-affected individuals. This suggests that it is unlikely that many restorations have masked enamel disturbances.

Our results showed that maxillary teeth in general were more often affected by MIH than mandibular ones, which is in accordance with most other studies (Leppäniemi et al. 2001; Preusser et al. 2007; Martínez Gómez et al. 2012). An exception is Parikh et al. (2012) who found that mandibular FPMs were statistically significantly more often affected than maxillary FPMs. In some papers, however, no such difference was reported (Jälevik et al. 2001; Chawla et al. 2008). The maxillary right FPM has previously been reported to be the most frequently affected

tooth (Lygidakis et al. 2008a; Martínez Gómez et al. 2012) among patients with MIH, which is consistent with the finding in the present study.

In agreement with previous studies, we found that opacities were the most frequent enamel defect in MIH teeth (Jasulaityte et al. 2007; Muratbegovic et al. 2007; Wogelius et al. 2008; Soviero et al. 2009; da Costa-Silva et al. 2010; Ghanim et al. 2011; Allazzam et al. 2014). On the other hand, the frequency of PEB and atypical restorations in FPMs in the present study was 24.3 and 18.8 %, respectively. This was higher than reported in Denmark (8.4 vs. 7.8 %) (Wogelius et al. 2008), Germany (12.7 vs. 9.6 %) (Petrou et al. 2015) and Iraq (24.0 vs. 3.2 %) (Ghanim et al. 2011), but more in line with the report from Saudi Arabia (34.8 vs. 8.7 %) (Allazzam et al. 2014). The surface of some opacities may break down when the tooth has been exposed to the oral environment for some time, and this may explain the relatively high frequency of PEB in the present study.

One typical feature of MIH is the non-symmetrical appearance in the dentition. This clinical experience is supported by the present results. Only 11 cases (10.0 %) showed a symmetrical distribution of enamel disturbances, which means that the same tooth on both sides was affected. Furthermore, the severity of the lesions varied between the corresponding teeth in the majority of these cases. The non-symmetrical occurrence of the enamel lesions in most MIH-affected individuals could suggest that the insult causing defective enamel is of short duration and affects ameloblasts at a critical phase (Fearne et al. 2004).

An interesting finding in the present population was the relatively high frequency of enamel disturbances in the canines (Fig. 3). In the maxilla, the canines were more often affected than lateral incisors. In addition a higher frequency and severity of affected canines in the maxilla than the mandible were found. A similar observation was recorded for the maxillary incisors, which were more often and more severely affected than incisors in the mandible in the present study. This is in line with previous reports (Preusser et al. 2007; Kühnisch et al. 2015; Petrou et al. 2015).

Experienced researchers working through the EAPD recommend that the optimal age for the clinical examination of MIH is eight years, while second primary molars should be examined at the age of five years (Elfrink et al. 2015). This is probably one reason why recent studies mostly focus on FPMs, incisors and second primary molars. The present cross-sectional study illustrated a more complete picture of MIH in the permanent dentition since assessment of canines was included (Liversidge 2000). In future research, a longitudinal design including examination in the adolescent period is recommended (Jälevik 2010; Lygidakis et al. 2010; Kühnisch et al. 2014).

Occlusal and buccal surfaces were most commonly affected in FPMs, as well as the labial surface in incisors, also reported by Petrou et al. (2015). These surfaces have thicker enamel than the lingual/palatinal surfaces in FPMs (Lygidakis et al. 2010). The thicker the enamel, the longer the formation period, which increases the possibility of enamel disturbances (Lygidakis et al. 2010). Another interesting phenomenon of MIH is that the cervical third of the tooth usually is not affected (Jälevik and Noren 2000; Preusser et al. 2007), which might be related to the thin enamel in this area.

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Paper II

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RESEARCH ARTICLE Open Access

Five-minute Apgar score ≤ 5 and Molar Incisor Hypomineralisation (MIH) – a case control study

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Abstract

Background: The aetiology of molar incisor hypomineralisation (MIH) is unclear. The asymmetric distribution of MIH in the dentition may indicate that an insult of short duration that affects ameloblasts at a vulnerable stage could be a causative factor. Apgar ≤ 5 at 5 min may indicate asphyxia (hypoxic-ischemic insult) during birth. It was hypnotised that low Apgar score during birth may cause MIH. The present study aimed to examine a possible association between Apgar ≤ 5 at 5 min and the occurrence of MIH.

Method: Two study groups were selected for examination. The cases comprised 67 children aged 8–10 years born with Apgar score equal to or below 5 after 5 min. The control group comprised 157 age-matched healthy children. First permanent molars, second primary molars and all permanent incisors were examined in all children. Clinical examination was undertaken by two calibrated examiners and intraoral close-up photographs of the teeth were later evaluated by three calibrated and blinded clinicians. Demarcated opacities, post-eruptive breakdown, atypical restorations and extractions due to MIH, according to the criteria of the European Association of Paediatric Dentistry, were assessed.

Results: The prevalence of MIH did not differ between the two groups. A chi-square test failed to confirm any statistically significant relationship between 5-min Apgar scores and MIH occurrence. In addition, there was no statistically significant relationship between the number of affected first permanent molars in cases and controls.

Conclusion: There was no association between Apgar ≤ 5 at 5 min and the occurrence of MIH.

Keywords: Ameloblasts, Asphyxia, Apgar score, Enamel, Molar incisor hypomineralisation

Background

The regenerative capability of dental enamel is fundamentally limited due to apoptosis of ameloblasts following maturation of the tissue. Disturbances in the function of ameloblasts during tooth development may therefore result in permanent defects. As development of the first primary tooth begins in the fourth week *in utero* and the root development of the wisdom teeth is completed around the age of 20 years, teeth serve a role similar to a "flight recorder" that covers a long time period. From this "record," the clinician can judge roughly when the disturbance occurred, and the appearance of the defects may in some cases give clues as to the aetiological factor. Thus, the tooth enamel often acts as a repository of information on systemic insults received during development [1].

Molar incisor hypomineralisation (MIH), as defined by Weerheijm et al. [2] in the early 2000s, describes a developmentally derived enamel hypomineralisation affecting 1 to 4 first permanent molars (FPMs) and frequently also permanent incisors. Clinically, the enamel defects of MIH present demarcated opacities that vary in colour from white to yellow/brown with a more or less sharp demarcation between the affected and sound enamel [3]. The asymmetrical occurrence of MIH molars within individuals suggests that the insult causing defective enamel is of short duration and affects ameloblasts at a

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critical phase [4]. Any temporary or permanent interruption of the ameloblast function depending on the time of insult, may cause enamel hypoplasia or hypomineralisation [4, 5]. Defects occurring in infancy can be diagnosed many years later when the teeth have erupted, as the enamel does not undergo remodeling. The aetiology is complex and there is insufficient evidence in the literature regarding possible factors that may cause these demarcated enamel defects. Some of these are environmental factors with systemic effects. These may include prenatal, perinatal and childhood medical conditions, but an underlying genetic predisposition cannot be excluded [6–8].

An old study of 102 children with neonatal asphyxia showed that 27 % had enamel defects compared with a control group ($n = 56$) with corresponding prevalence of 13 %. A possible relationship between neonatal asphyxia and enamel disturbances could not be ruled out, but the difference was not statistically significant [9]. A questionnaire study among parents of 10-year-old children in Iran revealed that 15 % ($n = 144$) of the children had serious illness during the first month after birth. In this group, the Apgar score < 7 was statistically significantly related with occurrence of developmental defects in permanent teeth (OR 2.32) [10]. The Apgar scoring system is used to assess the newborn immediately after delivery [11]. Low Apgar score may be indication of birth asphyxia. It has been suggested that the length of time it takes to reach Apgar score of 7 is a rough indication of severity of asphyxia [12]. Also low Apgar score at 5 min has been demonstrated to have some predictive value on neurodevelopmental outcome [13, 14] and a recent publication [15] showed that low Apgar score at 5 min was strongly associated with the risk of neonatal and infant death.

Maternal hypoxia during the latter stage of pregnancy has been demonstrated to disturb amelogenesis in the rat foetus [16]. Epidemiological studies indicate that the function of ameloblasts might be affected during human preterm birth [17, 18]. It cannot be excluded that low oxygen level plays a role in such cases. During asphyxia, the combination of the decrease in oxygen supply (hypoxia) and blood supply (ischemia) results in a cascade of biochemical changes that may lead to neuronal cell death and brain damage [19].

Animal studies have shown that hypoxic insult may result in both quantitative and qualitative defects in the enamel, reflecting the vulnerability of ameloblasts toward severe hypoxia. Rats subjected to hypoxia exhibited enamel aberrations in the form of hypoplasia [20], whereas both hypomineralisation and hypoplasia were observed in mice following acute hypoxic insult [21]. The asymmetric distribution of MIH in the dentition may indicate that an insult of short duration which affects ameloblasts in a vulnerable stage could be a causative factor.

The objective of this study was to test the null hypothesis that Apgar ≤ 5 does not increase the risk to develop MIH in humans.

Methods

The study protocol was approved by the Regional Committee for Medical and Health Research (REK) and informed consent was signed by each child's parents prior to data collection.

Sample selection

This was a cross sectional, case-control study, based on clinical and photographic examinations of 8–10 year old children ($n = 266$) (Fig. 1). Two groups of children were investigated, one group diagnosed with birth asphyxia defined as Apgar score ≤ 5 (Group 1) 5 min after delivery and one healthy control group (Group 2).

Group 1 (cases) comprised all children born 2004-6 admitted to the Neonatal Intensive Care, Ullevål, Oslo University Hospital, with an Apgar score ≤ 5 at 5 min. Originally, this group comprised 107 children of whom 80 were invited to participate (Fig. 1). The national population register (Folkeregisteret) was contacted to confirm the contact details obtained from the medical records. Group 2 (controls) comprised of children healthy at birth attending the Public Dental Service (PDS) in Lillestrøm in the county of Akershus. The controls were age-matched with the cases (born 2004-6). The exclusion criteria were children with genetic syndromes and malformations diagnosed in the neonatal period.

Data collection and analysis

A structured medical history questionnaire was sent or given to the parents of the participating children. The questionnaire included data on maternal health, pregnancy, delivery, prematurity, breastfeeding, use of antibiotics and infection during early childhood, as described in previous studies [22, 23]. The children were examined in the dental clinic by two dentists (AS and RS) for the presence and severity of MIH. Dental lighting, mirrors and blunt probes were used. When there was disagreement consensus was reached through discussion. Prior to the examination, a calibration exercise was conducted among all the examiners (RS, AS, ABS and IE) using clinical photographs of patients selected from the patient records in the Department of Paediatric Dentistry, University of Oslo. The examiners applied the diagnostic criteria that were agreed on at a workshop organised by the European Academy of Paediatric Dentistry [24]. All second primary molars and index teeth which include first permanent molars and all permanent incisors were

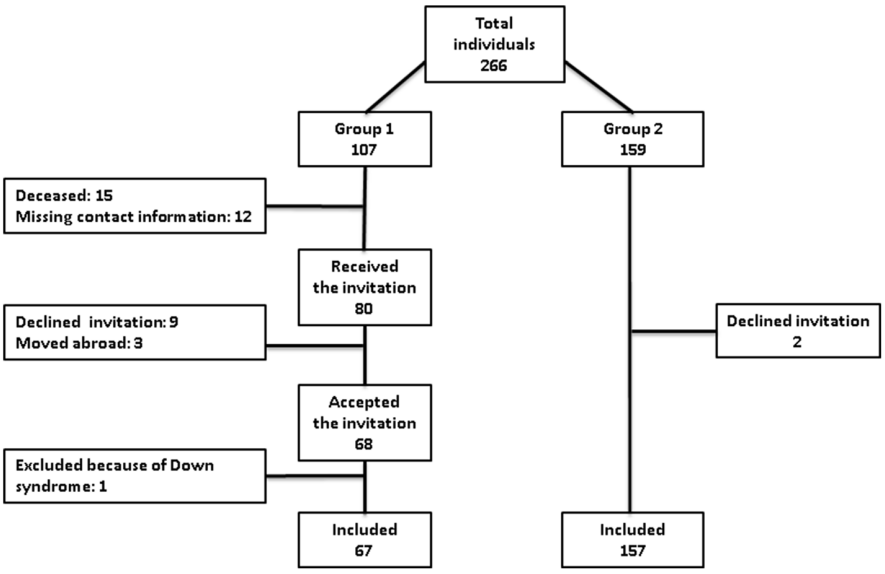

Fig. 1 Flow chart of individuals enrolled in the study

examined for demarcated opacity, post eruptive enamel breakdown (PEB), atypical restorations replacing affected enamel and extractions due to MIH (Fig. 2). Opacities < 1 mm in diameter were not recorded. The term Molar Incisor Hypomineralisation was used for dentitions with one or more hypomineralised FPMs with or without hypomineralised permanent incisors [25]. When more than one defect occurred on a tooth surface, the most severe was recorded. Sum scores of recordings on FPMs were calculated to express the severity of MIH in each individual. For each person with MIH, the sum score theoretically could range from 1 to 8, as each tooth received score 1 for opacity or 2 for PEB, atypical filling or extraction due to MIH. The sum score is the total of 4 single scores for each FPM. The final diagnosis of MIH was based on consensus among the observers. In some cases, the quality of the photographs was not optimal due to poor patient compliance and the clinical diagnosis was taken into account.

After the clinical examination, a digital camera (Canon EOS 60D, Canon INC., Japan) fitted with a macro-lens (Canon Macro Lens EF 100 mm 1:2,8 USM, Japan) and a ring flash (Canon Ring Lite MR -14EX, Japan) was used to capture macro-photographs of the dentition. Most of the photographs were taken by one photographer according to a standardised protocol. Seven photographs were taken in the following order: the buccal surfaces of the teeth in the first and fourth quadrant (#1), the corresponding surfaces in the second and third quadrant (#2), the buccal surfaces of the upper and lower anterior teeth (#3), and the occlusal surfaces of the upper teeth (#4 & 5) and lower teeth (#6 & 7). All the photographs were immediately examined for photographic quality, and if necessary photographs were repeated. The photographs were displayed on Sony 55" TV-monitor (Sony Model no.KDL 55WB02A LCD-TV, Sony Corporation, Japan) in a room with masked windows and dimmed room lighting. The intraoral photographs were then evaluated for enamel defects by three independent and blinded observers (AS, ABS and IE). One month later, the clinical photographs of a subsample of children ($n = 35$, 15.5 %) were re-examined in the same room under identical lighting conditions on the same TV monitor. Cohen's kappa was calculated for inter- and intra-examiner agreement.

Data handling

Data from questionnaires were tabulated and analysed using the Statistical Package for Social Sciences 22.0 for Windows (SPSS Inc., Chicago, Illinois, USA). Chi-square test was used for comparison. Significance was set at $p < 0.05$.

Results

Of 107 children in group 1, the parents of 54 children did not receive an invitation (Fig. 1). Eighty children/parents were eligible and therefore invited to take part in the study. Sixty-eight parents accepted the invitation (85.0 %). Out of the 159 controls invited to participate, two parents (1.3 %) declined (Fig. 1). The distribution of boys and girls in group 1 was 53.0 and 47.0 %,

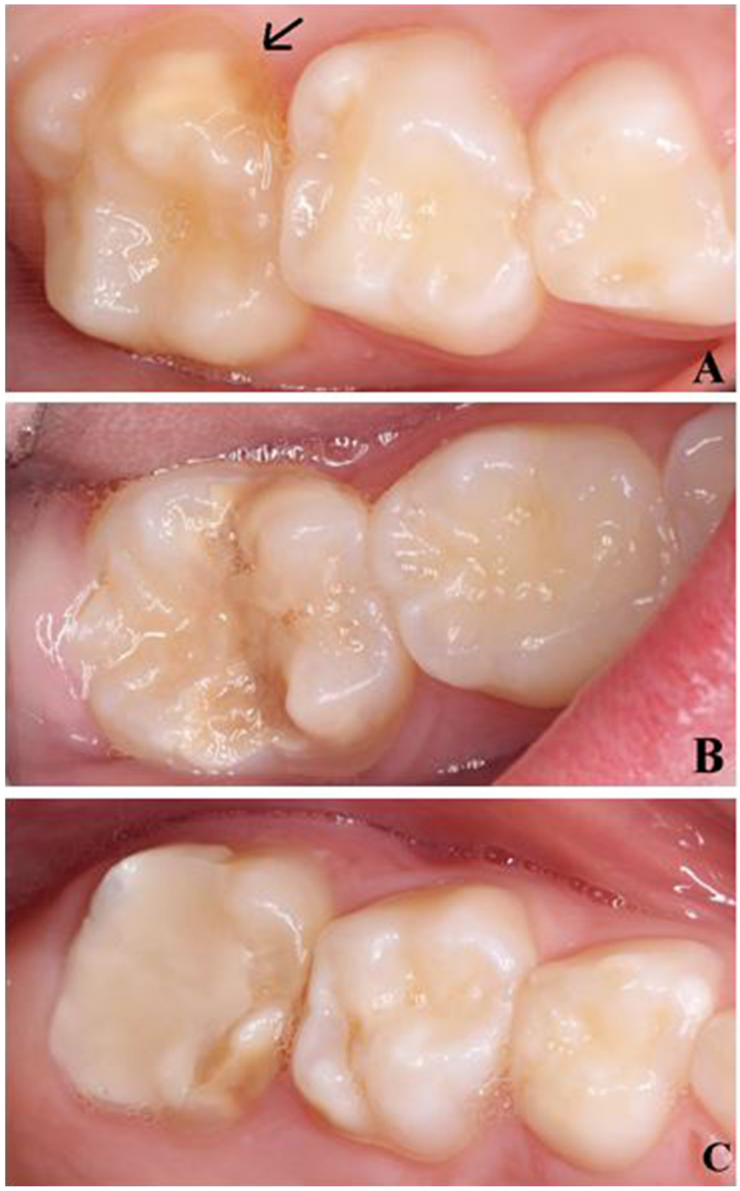

Fig. 2 Examples of affected first permanent molar from three individuals with MIH enrolled in the study. Defects varies in severity from diffuse yellow opacities (*black arrow*) in tooth 16 (**a**) to more severe changes due to posteruptive breakage (PEB) in tooth 46 (**b**) and atypical filling due to severe MIH in tooth 26 (**c**)

respectively and the corresponding rates in the control group were 49.7 and 50.3 %. The mean age in group 1 was 9.0 years (SD 0.84, range 8–10), and 9.0 years (SD 0.80, range 8–10) in group 2.

Based on the clinical examination, the prevalence of MIH in group 1 was 23.5 and 25.4 % in group 2 ($p = 0.76$). Some examples of MIH teeth found among the included individuals are shown in Fig. 2. The results of the joint decisions, based on examination of the intraoral photographs, showed that the MIH prevalence in the asphyxia group was 29.4 % and in the control group 31.2 % ($p = 0.79$). Furthermore, there was a higher proportion of controls (5.7 %) than cases (2.9 %) who had a MIH sum score between 5 and 8, but there was no statistically significant differences between the groups (Table 1) or in the average severity score per tooth between the two groups ($p = 0.42$). There were no statistically significant differences in the distribution of affected index teeth and second primary molars in individuals from both groups (Table 2). Analysis of

Table 1 Distribution of individuals according to MIH score in the Group 1 (Apgar score ≤ 5) and Group 2 (controls)

MIH score	Group		*p-value*
	Group 1 ($n = 68$)	Group 2 ($n = 157$)	
0	69.1	67.5	0.94
1–2	16.2	14.4	0.69
3–4	11.8	12.4	0.05
5–8	2.9	5.7	0.40
Total	100.0	100.0	

MIH score is cumulative score for all four FPMs in each individual. The scores for each tooth may vary from 0 (no MIH) to 2 according to severity. No statistically significant differences between groups

medical records of the children in group 1 who did not wish to participate showed that there was no increased morbidity during the first year of life.

There was no statistically significant difference in MIH prevalence between girls and boys neither in group 1 ($p = 0.75$) nor group 2 ($p = 0.57$). Inter-examiner and intra-examiner agreement for MIH diagnosis at the patient level, measured by Cohen's kappa, was in the range 0.60–0.78 (mean 0.69) and 0.55–0.76 (mean 0.65), respectively.

Analyses of the self-reported health information (questionnaires) revealed that there was no statistically significant association with MIH and health related variables between groups.

Discussion

The null hypothesis to be tested was that Apgar ≤ 5 at 5 min, does not increase the risk of developing MIH. The hypothesis was accepted as there was no statistically significant difference in the prevalence of MIH between the study group and controls based on clinical and photographic examinations. Further, the number of affected FPMs or the severity of MIH on affected teeth did not differ between groups and these findings support the rejection of the null hypothesis.

Table 2 Distribution of affected index teeth and second primary molars in individuals with Apgar score ≤ 5 (Group 1) compared to control individuals (Group 2)

Affected tooth group	Group		*p-value*
	Group 1	Group 2	
Upper FPM	22.8	21.3	0.63
Lower FPM	11.0	18.5	0.13
Upper central incisors	5.3	6.1	0.61
Upper lateral incisors	3.5	3.8	0.54
Upper second primary molars	8.2	5.4	0.43
Lower central incisors	2.9	2.5	0.64
Lower lateral incisors	3.0	2.6	0.77
Lower second primary molars	8.2	4.1	0.08

No statistically significant differences between groups

Many prevalence studies have been carried out in different countries using the new definition of diagnostic criteria for MIH [3] and this definition was also used in the present study. Large variations in prevalence have been reported, ranging from 2.4–40.2 % [26]. This wide range may be explained by differences in recording methods, different ages or different study populations [27, 28]. In some countries, generally high caries experience may mask the true prevalence of MIH [28]. The clinical examination showed a lower prevalence compared with recordings based on photographs. The prevalence of MIH in the present control group was found 25 %, based on the clinical examination, corresponds well with findings from Finland (25 %), Denmark (15–25 %) (Esmark 1995 cited by Weerheijm 2003 [25]) and Sweden (18 %) [29, 30]. However, these studies were published before the criteria by European Academy of Pediatric Dentistry were established [24] and some deviations according to variability in criteria applied might be expected. Digital photography with magnification on the monitor allows detailed examination of enamel [31], possibly explaining the higher prevalence reported with this method in the present study. In addition, the use of photographs facilitates randomisation and blinding, limiting observer bias, which is important in a study like this. As in other studies, there was no statistically significant difference in prevalence between the sexes in either group [32–34]. Furthermore, the relative distribution between tooth groups and upper and lower jaw are consistent with the findings that have been reported by Lygidakis et al. [35].

Decreases in oxygen supply are known to initiate adaptive mechanisms designed to maintain cellular activity at a minimum level. The failure of these mechanisms during hypoxia results in cellular dysfunction and can lead to irreversible cell damage [36]. In general, cellular adaptations to hypoxia rely on the transcription factor hypoxia-inducible factor (HIF), which is inactive when oxygen is abundant but is activated under hypoxic conditions [37, 38]. This may also be likely for the affected ameloblasts exposed to hypoxia. Further, it may be speculated that compensatory mechanisms like increased expression of HIF-1α, which is shown to be upregulated in ameloblast cells following hypoxia [39], may be the reason for not developing MIH in patients with mild hypoxia.

It is known that combined Apgar score and increased acidosis is more indicative of the severity of asphyxia. In 2004-2006, umbilical blood gases were not analysed routinely and in the present study, an Apgar score ≤ 5 at 5 min was used as entry criterion since it was used for

hospital admission at the time. Thus the hypoxia may not have been severe enough to induce changes in the ameloblasts to cause MIH.

Grahnén et al. (1969), in their study, were unable to demonstrate a significant difference in enamel hypoplasia of the primary teeth [9], but this study had some weaknesses: the cases included low birth weight children, while the controls did not. Furthermore, the criteria for diagnosis of asphyxia were not stringent.

Attrition of participants in group 1 could be due to unavailability, inaccessibility or dental anxiety. Some patients did not survive, which results in lack of information about enamel disturbances in this group. It may therefore be speculated that the results represent an underestimation of MIH. It is also tempting to suggest that the hypoxia was a momentary insult that possibly affected the ameloblasts in a very short but vulnerable stage, which clinically appears in the form of asymmetrical defects influencing the FPMs [40]. This is in accordance with our recent findings demonstrating that there is a considerable variation in ameloblast reaction to hypoxia [21].

Several studies have suggested that hypoxia may affect ameloblast function adversely, both in the rat [16, 41], the hamster [42] and human [17, 43–46]. The results from the present clinical investigation were not consistent with our findings recently reported in an animal study [21]. We have previously demonstrated that a short episode of induced severe hypoxia in adult mice left its mark on mouse incisors in the form of enamel defects. The position and character of the defect were related to the functional stage of the affected ameloblasts. All the cells of the enamel organ, i.e. preameloblasts, secretory ameloblasts, transitional ameloblasts, maturation ameloblasts and pigmentation ameloblasts were subjected to the hypoxia, but seemed to respond differently. Affected enamel showed hypoplasia with or without hypomineralisation [21].

Conclusions

Based on the results of the present cross-sectional, case-control study, it can be concluded that a low Apgar score at 5 min was not associated with the appearance of MIH or the number of affected FPMs. Bearing in mind that this was probably a mild insult, further investigation of individuals with lower Apgar score would shed more light on the role of hypoxia in the etiology of MIH. In future studies low Apgar score should be associated with other clinical markers of asphyxia like increased acidosis.

Abbreviations

FPMs, first permanent molars; HIF, hypoxia-inducible factor; MIH, molar incisor hypomineralisation; PEB, post eruptive enamel breakdown; PDS, Public Dental Service; REK, Regional Committee for Medical and Health Research

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Availability of data and materials

Data will not be shared because that was not a part of the agreement with the Oslo University Hospital and according to the information to the parents.

Authors' contributions

RS and ASch carried out the clinical and photographic examinations. RS, ASch, ABS, Aseh, TS and IE participated in the analysis and interpretation of the data. All the authors participated in the design of the study in addition to read and approve the final manuscript.

Competing interests

The authors declare no conflict of interest for this study.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The study protocol was approved by the Regional Committee for Medical and Health Research (REK) and informed consent was signed by each child's parents prior to data collection. The committee's reference number is 2013/1072-5.

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ORIGINAL SCIENTIFIC ARTICLE

Effects of antibiotics on the developing enamel in neonatal mice

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Abstract

Purpose Identifying factors causing Molar-Incisor Hypomineralization (MIH) is an ongoing challenge. Preterm infants, routinely treated with antibiotics in cases of suspected sepsis, are more commonly affected by dental developmental defects. This study aimed to investigate the effects of gentamycin and ampicillin on the developing enamel in neonatal CD-1 mice in vivo.

Methods Neonatal mice were randomized into a study ($n=36$) and a control ($n=35$) group. Antibiotics were injected intravenously for 4 days. All mice were sacrificed after 15–18 days. Micro-CT was used to analyse the mineral density (MD) of the enamel and the proportion of the enamel object volume (vol%) in first molars and incisors.

Results We demonstrated a significantly lower vol% enamel in the maxillary (30.9% vs. 32.7%; $p=0.004$) and mandibular (32.5% vs. 34.6%; $p=0.015$) molars in the study group than in the controls. The incisors were divided into segments upon analysis. We demonstrated both lower vol% and lower MD of the enamel in most segments in treated individuals compared to controls ($p<0.05$).

Conclusion The reduced MD and vol% in the molars and incisors are likely to have been caused by the antibiotics given during tooth development. The presented analysis of teeth in neonatal mice with micro-CT could be a valid model for further research on dental developmental defects.

Keywords Dental enamel hypoplasia · Molar-Incisor Hypomineralization · X-ray Microtomography · Tooth · Gentamicin · Ampicillin

Introduction

Dental enamel, covering the tooth crown, is produced by ameloblast cells. It is a unique tissue that exhibits remarkable wear and fracture resistance (Simmer et al. 2010). The aetiology of developmental enamel defects is multifactorial and provides challenges for basic science research and clinical management (Seow 2014). Molar-Incisor Hypomineralization (MIH) is one of the most common dental developmental disorders and affects one to four first permanent molars (FPMs). Permanent incisors are also frequently involved (Lygidakis et al. 2010). In Scandinavia, the prevalence of MIH varies from 13.9% in Northern Norway (Schmalfuss et al. 2016) to 37.3% in Denmark (Wogelius et al. 2008). Patients who are severely affected by MIH constitute a challenge in paediatric dentistry due to commonly occurring hypersensitive teeth and an increased risk of decay (de Souza et al. 2016). Repeated operative treatments may result in clinical behaviour management problems, dental fear and anxiety (Jalevik and Klingberg 2002). Our knowledge of the aetiology of MIH is still elusive. Locally disrupted amelogenesis has been suggested. Disturbances in tooth development must occur within the last trimester of pregnancy and up to the third year of a child's life to result in MIH (Weerheijm and Mejare 2003). Several possible factors causing MIH have previously been reported (Garot et al. 2021). However, for most of them, the evidence is weak or absent. Environmental pollution, e.g., dioxins and polychlorinated biphenyls (Alaluusua et al. 1996), genetic predisposition (Jeremias et al. 2013) and several different conditions and diseases, including their treatments, have been suggested as aetiological factors. Several studies have associated a higher prevalence of MIH with serious illness during early childhood or complications, especially during the last trimester

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of pregnancy (Garot et al. 2021). A crucial question is whether enamel defects are caused by the illness itself, medications or a combination of both (Laisi et al. 2009).

The frequent use of antibiotics for the treatment of common childhood diseases, such as acute otitis media and respiratory infections, is potentially associated with MIH (Allazzam et al. 2014). Prematurity (< 38 weeks) (Mejia et al. 2019) or very low birthweight (LBW) (< 2500 g) (Ghanim et al. 2013) have also been associated with an increased occurrence of enamel defects in primary and permanent dentition. Brogardh-Roth et al. (2011) showed that MIH was more than twice as common in preterm infants and that LBW was a concomitant factor. These findings were confirmed by de Lima Mde et al. (2015). Furthermore, sepsis is one of the most common complications in premature infants. The recommendation by the World Health Organization for the treatment of preterm infants at risk for sepsis is followed by Norwegian hospitals, which administer ampicillin in combination with gentamicin (Fuchs et al. 2016). This treatment is given to the majority of preterm infants and has led to a significant reduction in mortality in premature and LBW infants (Bizzarro et al. 2005). Overprescription of antibiotics for fear of potentially dramatic consequences from a delayed neonatal sepsis diagnosis is also suspected based on empirical indications in many uninfected neonates (Fjalstad et al. 2016). Although the use of antibiotics during enamel formation in children has been associated with the aetiology of MIH (Serna et al. 2016), experimental evidence is scarce. The use of amoxicillin in early childhood has been associated with MIH (Laisi et al. 2009). An in vitro study demonstrated that amoxicillin affected enamel formation in cultured mouse embryotic tooth explants (Sahlberg et al. 2013). A recent in vivo study using relatively higher doses of amoxicillin showed a decrease in electron density in some rats, but the difference was not statistically significant (Feltrin-Souza et al. 2020).

To increase our understanding of how antibiotics other than amoxicillin affect enamel formation, ampicillin and gentamicin were administered to neonatal mice in doses similar to those given to LBW infants, and their effects on the enamel of molars and incisors were studied (Fuchs et al. 2016). The aim of this study was to determine whether the use of ampicillin and gentamicin in combination could cause mineralization disturbances in dental enamel.

Materials and methods

Animal model

Five pregnant mice (CD-1 strain) were maintained on a 12 h light–dark cycle at 21 °C and a relative humidity of 55%. The mice were given standard laboratory fodder and water ad libitum. The animals were kept according to the regulations of the Norwegian Gene Technology Act of 1994. In addition, they were kept in calm surroundings and an enriched environment facilitated with a variety of 'toys' to minimize suffering and distress. The experiment was approved by the Norwegian Local Veterinary Service (FOTS ID 8325). This study conforms to the ARRIVE guidelines.

From each litter, the neonatal mice ($n = 71$) were randomly allocated to a study group ($n = 36$) and a control group ($n = 35$). Based on the study by Sorensen et al. (2007) demonstrating that tail tip amputation only had minor short-term negative effects on mouse welfare, we performed this method to mark the mice of the control group. Starting at postnatal day 1 (P1) or 2 (P2), drugs (described later) were intravenously injected for 4 days in the morning as described by Glascock et al. (2011). Filtered green food dye (1:100) was added to the solutions to control access to the intravenous injection. The green dye rendered the drug distribution visible, as the normal pink colour of the neonates was converted to green. Before injection, the facial vein was made visible using a Wee Sight™ transilluminator (Philips, Amsterdam, Netherland). The solution was slowly injected with an insulin syringe (Micro-Fine™ 8 mm × 30 G; Becton Dickinson, New Jersey, USA) using a 2.5× magnifying visor loupe (Lactona, Bergen op Zoom, Netherland). Bleeding from the injection site was stopped by applying pressure with a gauze swab. After the transient stress from the injection was relieved, the mice were returned to the cage and kept under standard conditions. All treatments were performed by experienced, specially trained personnel, and all efforts were made to minimize suffering. The animals' health and behaviour were monitored every day during the treatment period and every second day afterwards. According to the research protocol, all mice with health deficits or unnatural behaviour would have been euthanized immediately.

All neonatal mice were sacrificed by cervical dislocation according to the Norwegian Local Veterinary Service protocol at the age of P16 to P18. The jaws, including incisor and molar teeth, were dissected out and fixed in 70% ethanol. After fixation, the jaws were thoroughly cleaned by gentle brushing in running tap water using a stereomicroscope with a light source (SteREO Discovery. V8 & SteREO CL 1500 ECO; ZEISS, Oberkochen, Germany). Teeth with visible iatrogenic decay accidentally inflicted during preparation were excluded from the study.

All medicines were purchased from the hospital pharmacy (Sykehusapoteket Rikshospitalet, Oslo, Norway) and prepared for the study group as follows: the ampicillin solution was prepared by mixing powder and sterile physiological saline according to the manufacturer's instructions (Pentrexyl; Bristol-Myers Squibb, Lysaker, Norway) and adding sterile filtered green food dye (1:100). Gentamicin (Gentamicin; B. Brown, Melsungen, Germany) was administered

using a prefabricated solution (3 mg/ml) for injections. Before use, both antibiotic solutions were mixed according to the requested dose: ampicillin solution (200 mg/kg) 2.0 µl/g mouse body weight and gentamicin solution (4 mg/kg) 1.3 µl/g mouse body weight, equivalent to what is given to preterm infants in Norwegian hospitals. The injected volume per mouse was between 6.6 µl and 15.2 µl, corresponding to the body weight (2.0–4.6 g). For the control group, sterile saline was prepared with sterile filtered green food dye (1:100), and a volume corresponding to 3.3 µl/g mouse body weight was injected.

Macro photography

As a part of the enamel examination, photographs were taken using a digital single lens reflex camera with a macro lens (D700 & AF Micro Nikkor 60 mm, 1:1; Nikon, Tokyo, Japan) and extension tubes (DG 12, 20 and 36 mm; Kenko Tokina, Tokyo, Japan) with standardized camera settings (ISO 200, f32, 3 s): the labial aspects of the upper and lower incisors and the occlusal surfaces of the first upper and lower molars. The photographs of 44 mice included in the study were projected onto a flat screen in a room with indirect, standardized lighting and examined individually and independently by three experienced dentists (ASc, ASe, IJB). The pictures were randomized and blinded before the examination. Enamel disturbances, such as demarcated opacities (white, yellow and brown colour), hypoplasias and other enamel defects were recorded. A joint score was decided for each recording, and consensus was reached through discussion when individual examiners differed.

Micro-CT imaging

A micro-CT scanner (SkyScan 1172; Bruker, Brussels, Belgium) was used to provide three-dimensional images of mouse incisors and first molars to determine the enamel volume and density (de Lima Mde et al. 2015). The scanning protocol and parameters for human teeth described in previous studies (Johnsen et al. 2016) were used with adjustments to the requirements for analysis of murine dentitions. Samples were mounted vertically in customized tubes. Hydroxyapatite phantoms with known density were used to calibrate the instruments. The scanning parameters were 4.96 µm isotropic pixel size with a medium camera resolution and X-ray source (100 kV, 100 mA, 10 W) using a 0.5 mm Al filter. Samples were rotated 360° around their vertical axis, with a 0.4-rotation step and frame averaging of three. A flat field correction was performed before every scan. The X-ray attenuation coefficients were reconstructed with SkyScan software Nrecon (Bruker, Brussels, Belgium) to serial coronally oriented tomograms using a modified algorithm, with beam hardening of 20%, ring artefact correction of 12 and an attenuation coefficient range of 0.00–0.05. The greyscale was first defined using control mouse enamel, allowing the isolation of enamel alone without any other structure. The resulting histogram was used to determine a binary threshold of 60–255 for hard tissue, including enamel and dentine, and 130–255 for isolated enamel.

Before analysing the enamel, first molars and incisors were isolated from the surrounding tissue prepared digitally using DataViewer (Bruker, Brussels, Belgium). The crown of the molars was isolated from the root at the cementoenamel junction in a standardized way (Fig. 1). The incisors were divided into six segments, each being 0.7 mm long starting at the incisal edge with S1 and ending at the most apical part of the tooth representing S6 following a standard protocol (Fig. 1). All images were inspected visually and with micro-CT, and iatrogenic artefacts were registered.

SkyScan software CTan and CTvol (Bruker, Brussels, Belgium) were used to determine enamel quality and quantity. The enamel was analysed according to mineral density (MD) (de Lima Mde et al. 2015) and the proportion of the enamel object volume (vol%) of the total hard dental tissue of the tooth or segment (Fig. 1).

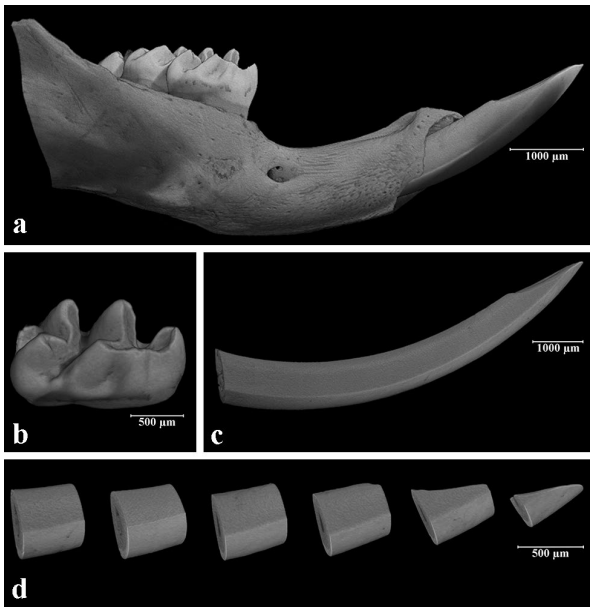

Fig. 1 Three-dimensional reconstruction of slices from micro-CT of 18-day-old mice from the study group: **a** right mouse mandible, buccal view; **b** isolated crown of the first mandibular molar; **c** isolated crown of the mandibular incisor; **d** mandibular incisor divided into six segments (S1–S6)

Statistical analysis

Results (enamel volume and density) are expressed as the mean ± SD of independent experiments in the upper and lower first molars and in every single segment (S1 to S6) of the incisors. Shapiro–Wilk's test was used to test normal distribution. If normality failed, values were transformed using log10, $\sqrt{X}$ or 1/X, and Shapiro–Wilk was repeated. One-way ANOVA was used to analyse the variance.

Welch's *t* test was performed in cases, where both normality and variance failed. Student's *t* test was used in cases with normal distribution and equal variance. The Mann–Whitney *U* test was performed when normality failed and the variance was equal. Groups were considered significantly different if $p < 0.05$. All calculations were carried out using SPSS version 24.0 (IBM SPSS Inc., Chicago, USA).

Results

The flow chart of animals and teeth included in the final evaluation step of the study is presented in Fig. 2. None of the mice died directly under or immediately after the injection; however, during the observation period until P16 to P18, 18/36 mice in the study group and 9/35 mice in the control group died within P6 (Fig. 2). Unfortunately, these mice could not be examined due to natural animal cannibalism. The mean (SD) age of the mice at the final point was 16.9 (± 0.78) days. Among the included mice, 14 first molars and incisors (7.9%) were excluded due to iatrogenic decay. In total, 18 mice from the study group with 65 first molars and incisors were included. In the control group, 26 mice with 97 first molars and incisors were evaluated (Fig. 2).

When dividing the incisors into six equal segments, the apical part of the enamel at approximately S5–S6 was less mineralized than the enamel closer to the incisal edge (Fig. 3). This made the enamel more vulnerable during the dissection process, and in two cases, the most apical segments (S6) were excluded because of enamel fractures.

No differences between the study group and the control group were observed by visual examination of macrophotographs (Fig. 4). The enamel vol% in the first molars was significantly lower in the study group than in the control group in both the upper (30.9% vs. 32.7%; $p = 0.004$) and lower (32.5% vs. 34.6%; $p = 0.015$) jaws (Fig. 5). The vol% of the study group was significantly lower in the mandibular incisors ($p < 0.05$) in all segments (S1–S6) of the mandibular incisors. In the maxilla, two incisor segments (S3, S4) showed a significantly lower vol% compared to the controls (Fig. 6). The MD of the enamel from the study group was significantly lower ($p < 0.05$) in four segments (S1–S4) from the maxillary incisors than that of the control group. In the mandibular incisors, the MD of the enamel was significantly lower in segments S1, S2, and S4 (Fig. 3). There was no significant difference between the MD of enamel from any of the first molars when comparing the study and the control groups.

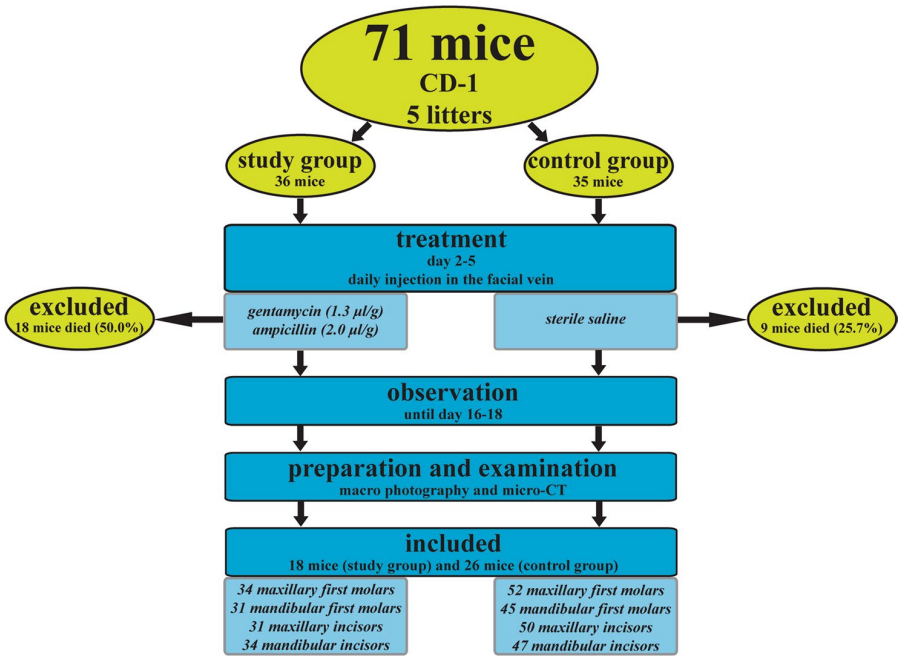

Fig. 2 Flow chart of animals enrolled in the study

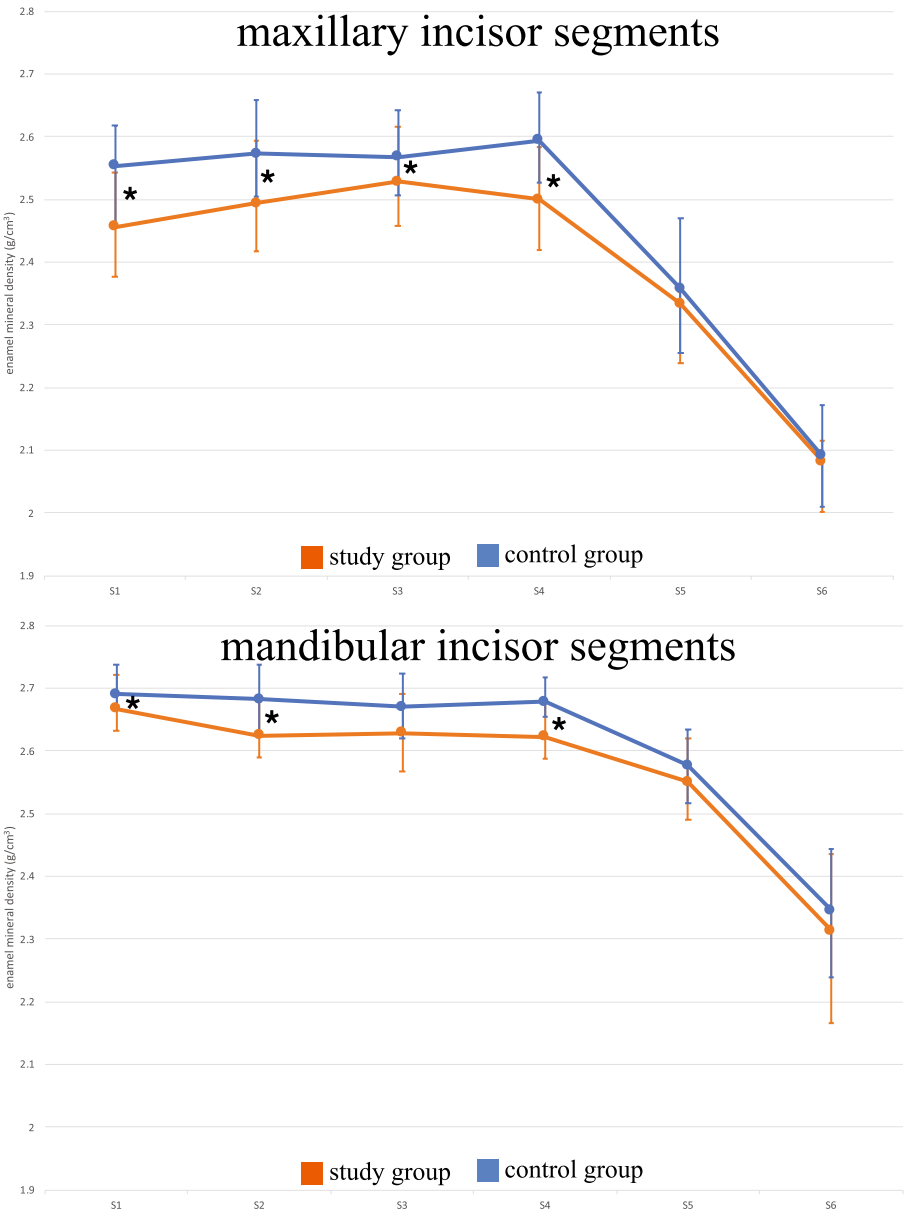

Fig. 3 Median and interquartile range of the enamel mineral density (g/cm^3) of maxillary and mandibular incisor segments (S1–S6); *significant difference between the study group and the control group ($p < 0.05$)

Discussion

Disturbances in enamel mineralization can affect both quality and quantity. In this study, we demonstrated that therapeutic doses of the antibiotics ampicillin and gentamicin reduced incisor MD values; similar alterations were observed in MIH. In contrast, the reduced enamel volume, which was demonstrated to indicate hypoplasia-like defects, is not consistent with MIH. In MIH-affected human molars, the MD values have been reported to be approximately 20% lower than those in clinically sound teeth, while the quantity of enamel was not affected (Fearne et al. 2004).

Studies on antibiotics and tooth development reveal conflicting results. Previous research using embryonic mouse molars in vitro showed that enamel was dose-dependently thicker in explants exposed to amoxicillin for 10 days (Laisi et al. 2009). The authors speculated that amoxicillin interfered with the function of ameloblasts by advancing the initiation of amelogenesis or the enamel accretion rate and concluded that this could explain the hypomineralization in MIH. Recently, temporarily significantly thinner enamel in rats treated pre- and postnatally with amoxicillin has been demonstrated by de Souza et al. (2016). Gottberg et al. (2014) showed that enamel thickness was not affected in rats treated prenatally with amoxicillin, but dose-dependent

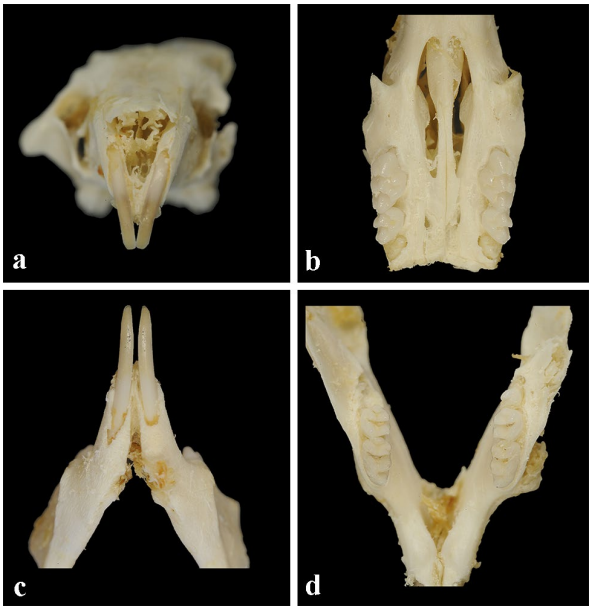

Fig. 4 Photographs of teeth from mice in the study group: **a** maxillary mouse incisors; **b** maxillary mouse molars; **c** mandibular mouse incisors; **d** mandibular mouse molars

enamel hypomineralization was observed. This finding was confirmed in mice, and the authors concluded that chronic exposure to amoxicillin/clavulanic acid in doses used in humans caused hypomineralized enamel (Mihalas et al. 2016). Another study did not show any effect on enamel after amoxicillin treatment in rats (Kumazawa et al. 2012). It was suggested that amoxicillin affects the expression of the metalloproteinase MMP20, which has an important role in the degradation and removal of enamel proteins (Sahlberg et al. 2013). However, these findings were not confirmed by de Souza et al. (2016). In the present study, the antibiotics used were gentamicin and ampicillin administered intravenously, consistent with the treatment of human preterm infants diagnosed with sepsis. Due to filial infanticide and cannibalism, the cause of mortality in the present study remains unknown. It could be speculated that the medication used was close to the lethal dose. However, gentamicin, well known for its nephrotoxicity, has previously been used in mice at considerably higher doses without inducing toxic side effects (Blakley et al. 2008). It could also be speculated that the intravenous injection technique was too traumatic for some of the neonatal mice; however, care was taken to avoid injuring the mice. In other in vivo animal studies, antibiotics have been administered orally, intravenously, subcutaneously, intramuscularly and intraperitoneally. It was assumed that if partial paravenous or subcutaneous administration occurred, the dose was likely to be equally effective. Drug levels in blood serum or plasma were not measured to avoid additional burden on the animals. To the best of our knowledge, gentamicin and ampicillin have not yet been tested in isolation or in combination for their effect on enamel mineralization in vivo. In this study, the effects of these antibiotics were able to be examined in isolation without any confounding factors, such as infections seen in preterm infants treated with antibiotics.

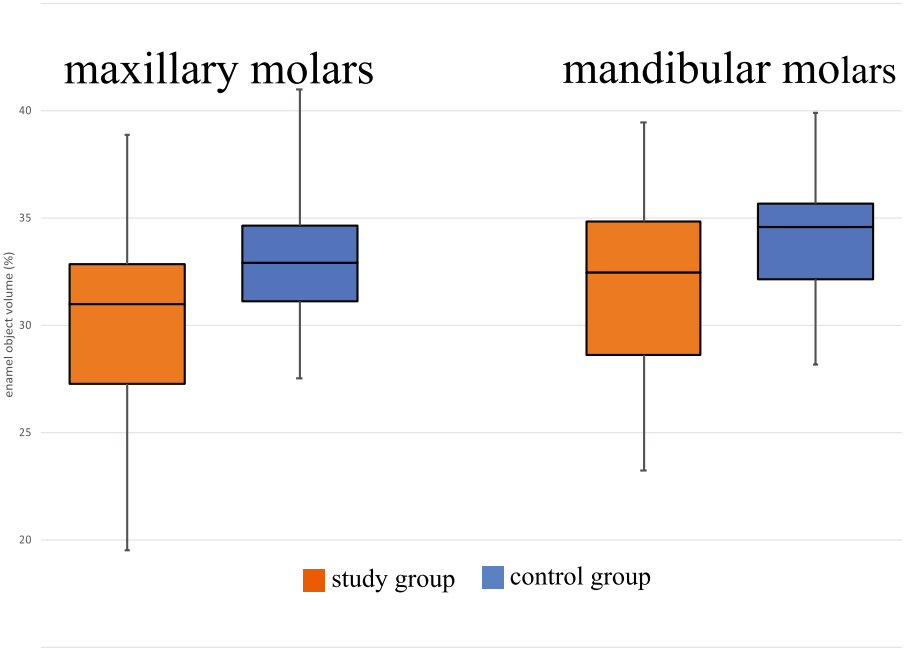

Fig. 5 Enamel object volume (%) in the maxillary and mandibular first molars. There were significant differences between the study group and the control group in both the maxillary ($p = 0.004$) and mandibular teeth ($p = 0.015$)

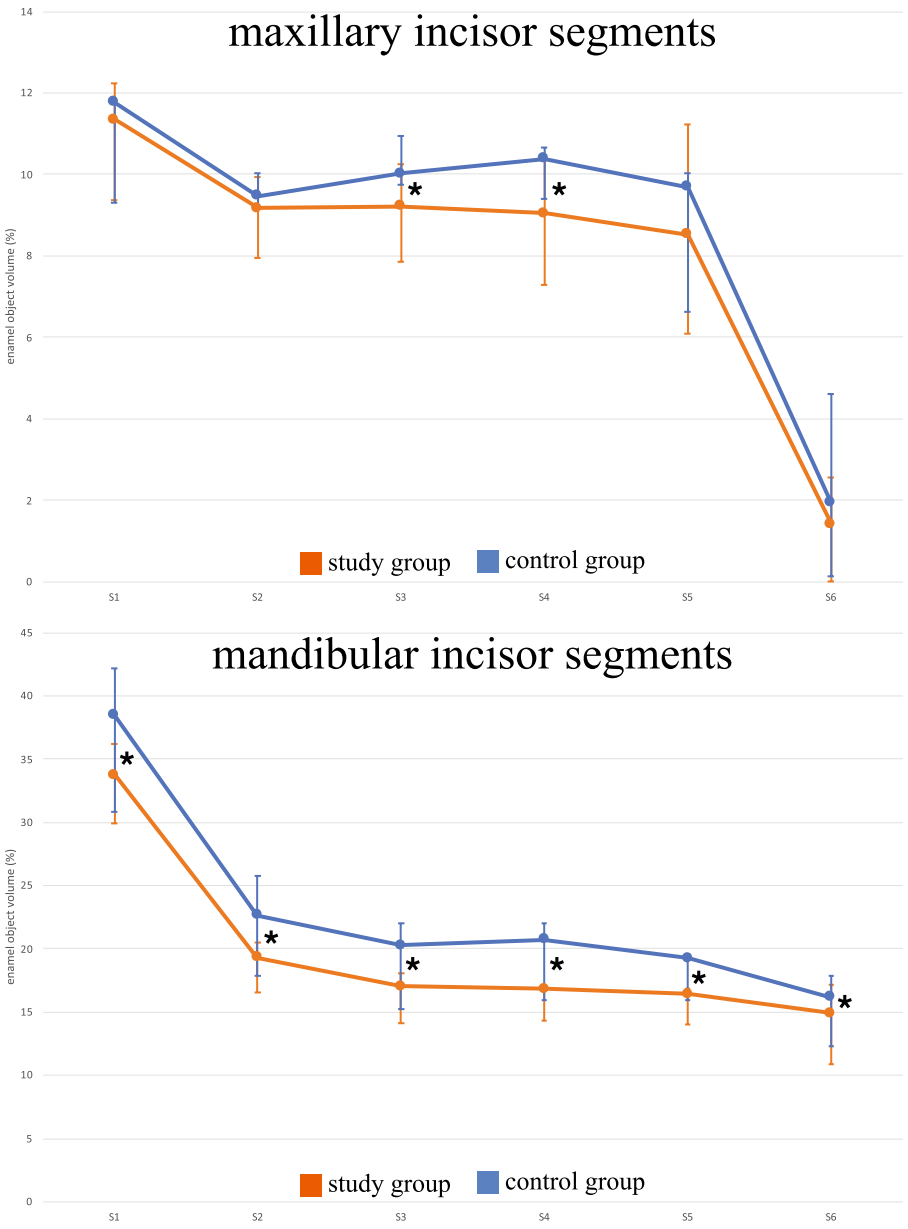

Fig. 6 Median and interquartile range of the enamel object volume (%) in maxillary and mandibular incisor segments (S1–S6); *significant difference between the study group and the control group ($p < 0.05$)

In enamel research, micro-CT is a commonly accepted noninvasive technique to evaluate enamel density and volume representing hypomineralization and hypoplasia. In contrast to analyses with a significantly higher resolution, such as scanning electron microscopy (SEM) or quantitative backscattered electron (qBSE) imaging, micro-CT is not dependent on ground sections. Making accurate longitudinal ground sections of mouse mandibular incisors is difficult due to the small size and slight medio-lateral curvature of the teeth (Sidaly et al. 2015). While measurement of the enamel thickness in ground sections of teeth is limited to the selected slide, micro-CT enables a three-dimensional analysis of the enamel in the selected area (Fagrell et al. 2013). Enamel defects can be detected independently of their various tooth locations. In micro-CT, the enamel is detected and separated from dentine by its density. Severely hypomineralized enamel may be misinterpreted as dentine by micro-CT, leading to volume measurements of enamel being lower and those of dentine being higher. This bias was prevented by a visual examination of both clinical pictures and micro-CT images, where misinterpretations could have been registered (data not shown).

The difference in MD of enamel between the study and control groups was less than 4% in the present study. The

entire section, i.e., both sound and affected enamel, was analysed. Due to the size of human molars, affected enamel can be analysed separately, which may result in greater differences (Fearne et al. 2004). The local temporary areas of hypomineralized enamel shown by Lyngstadaas et al. (1998) using SEM were not seen in the present study. It might be that micro-CT may not be sensitive enough to visualize these microscopic hypomineralizations.

Studies have shown that in first molars, ameloblasts at the tips of future cusps enter the secretory phase just after birth (P0), while at P2, crown mineralization advances by involving more ameloblasts in enamel production. On the fourth day (P4), dental crown morphogenesis is completed (Lungova et al. 2011). However, different strains of mice have been used in these studies, and differences in timing should be expected. Whereas Lungova et al. (2011) described a partially erupted first molar on day 16, all first molars were fully erupted in the present study. In the present study, medical treatment was performed from days P2 to P5; therefore, the maturation stage of enamel morphogenesis—but probably not the initial phase of amelogenesis—could be affected.

The eruption and enamel secretion rate of mandibular incisors (200 µm/d; 4.4 µm/d) is higher than that of maxillary incisors (140 µm/d; 7.3 µm/d) in adult mice (Sidaly et al. 2015). Slightly slower tooth development resulting in thinner enamel is expected in neonates (Schour and Massler 1967). In molars, secretion of enamel (7 µm/d) takes approximately 15 days with a final enamel thickness of approximately 100 µm (Lyngstadaas et al. 1998). In the present study, the enamel volume of the first molars was significantly reduced in the mice receiving drugs, which indicates that the secretory phase of amelogenesis is affected. This is consistent with results showing thinner enamel in rats treated with amoxicillin during the secretory stage of amelogenesis (de Souza et al. 2016). Smaller tooth size has been reported in preterm children and children with low body weight (< 2500 g) (Schuurs 2012). In human FPMs, the initiation of mineralization starts 1–7 weeks before birth (Antoine et al. 2009), while in mice, mineralization starts on day P2. This means that the onset of mineralization of FPMs in preterm infants is comparable to that in mice born to term.

Mice are convenient and extensively used for enamel research because of their comparable enamel structure. They are frequently used to examine the effect of medicine or environmental toxins on amelogenesis, but the potential for differences in pharmacokinetics between neonatal humans and mice remains uncertain (Warshawsky et al. 1981). In contrast to humans, mice have just a single dentition, probably the equivalent to human deciduous teeth (Tucker and Sharpe 2004). Although the present study aimed to explore MIH in permanent human dentition, similar defects exist in the primary dentition (Elfrink et al. 2012).

In contrast to incisors, mouse molars form a fast-developing model for odontogenesis in human molars. The mean formation time of the crown in human FPMs is more than 3 years (Reid and Dean 2006), while it is approximately 3 weeks in mice (Lungova et al. 2011). This quick sequence of stages in mice may make a direct comparison difficult (Fejerskov 1979), especially for studies of the transition stage (between secretion and maturation), which is assumed to be most vulnerable for the ameloblasts (Fearne et al. 2004). In the present study, mice were preferred to rats because of the slower eruption rate of the incisors, the longer enamel secretion phase and the greater enamel–dentine ratio in the former (Moinichen et al. 1996).

It is important to mention that the extrapolation of findings from the present animal study to humans must be conducted carefully. Rodent and human enamel has the same basic structural elements, prism and interprism; however, the spatial arrangement of prisms is considerably different (Warshawsky et al. 1981). Other differences include the speed at which enamel formation occurs and the incorporation of iron in the superficial enamel layer of rodent incisors (Risnes 1979). In the present study, we aimed to perform mouse injections with doses used in humans. However, the possible limitation of the present study may be that the drug uptake in rodents is different and/or that even the injection volumes/technique in such small animals may have varied. All these factors may have had an impact; however, we believe that this may not have significantly affected the major findings of our study.

Conclusions

Considering the limitations of the present study, the following conclusions can be made: The intervention given to the neonatal mice in the present study was timed to influence the developing molars and incisors. The reduced MD and volume in the first molars and the erupted part of the incisors are likely to have been caused by antibiotics. The presented analysis of teeth in neonatal mice with micro-CT could be a valid model for further research on MIH. As the research on the effect of antibiotics on enamel development do not show conclusive results, further research is needed.

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Data availability All data are fully available without restriction. The data underlying the results presented in the study are available from the author Andreas Schmalfuss.

Declarations

Conflict of interest All authors state that they have no conflicts of interest.

Ethical approval All authors gave their final approval and agreed to be accountable for all aspects of the work. Vertebrate animals (mice) were used. The animals were kept according to the regulations of the Norwegian Gene Technology Act of 1994, and the experiment was approved by the Local Veterinary Service (FOTS ID 8325). This study conforms to the ARRIVE guidelines.

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